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Application of:	Weinstock and Elliott		
Serial No.:	09/362,598	Examiner:	R.A. Zeman
Filed:	July 28, 1999	Art Unit:	1645
Entitled:	USE OF PARASITIC BIOLOGICAL AGENTS FOR PREVENTION AND CONTROL OF AUTOIMMUNE DISEASES	Conf. No.:	7062

Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF JOEL V. WEINSTOCK AND DAVID E. ELLIOTT UNDER 37 C.F.R. § 1.132

I, Joel V. Weinstock, hereby declare that:

1. I am one of the inventors in the above-named patent application. I am employed by the University of Iowa as a Physician-Scientist in the Department of Internal Medicine. I received a M.D. from Wayne State University in 1969. I have been employed by the University of Iowa since 1986.

I, David E. Elliott, hereby declare that :

2. I am one of the inventors on the above named patent application. I am employed by the University of Iowa as a Physician-Scientist in the Department of Internal Medicine. I received a Ph.D. in Immunology/Microbiology from Wayne State University in 1985. I received a M.D. from Wayne State University in 1988. I have been employed by the University of Iowa since 1991.

3. The instant application is directed to a method of screening a helminthic parasite preparation for one or more components that reduce a Th1 immune response, the method comprising the steps of: (a) obtaining a helminthic parasite preparation; (b) producing a homogenate of the helminthic parasite preparation; (c) separating fractions of the homogenate; (d) assaying a fraction of the homogenate to determine whether the fraction decreases a Th1

immune response; wherein a decrease in a Th1 immune response is indicative of the fraction comprising one or more components that reduce the Th1 immune response; and (e) further fractionating the fraction of step (d) into sub-fractions and identifying a sub-fraction that reduces a Th1 immune response.

4. We recognize that iterative fractionation and testing of resulting fractions and sub-fractions for activity, as claimed, is a well-known and routine method for isolating the biologically active component(s) of a complex biological mixture. It is also well known in the art that the same assay can be used at each stage of a fractionation procedure to monitor which fraction(s) or sub-fraction(s) have the activity of interest. For example, when fractionating an enzyme-containing preparation, the same enzyme assay is most often used at each stage of the fractionation procedure to monitor which fraction(s) or sub-fraction(s) contains the activity.

5. The Exhibits attached herewith (Exhibit A-C) further demonstrate that iterative fractionation and testing of resulting fractions and sub-fractions for activity, as claimed, is well-known and routine. Although the ultimate goals of the cited reference were to purify various proteins, all references describe similar fractionation processes. The references clearly establish that the fractionation and testing of resulting fractions and sub-fractions for activity is a well-established practice in the art.

6. In Palczewski et al. (1988, J. Biol. Chem 263: 14067-14073; Exhibit A), an iterative sub-fractionation approach is used to purify rhodopsin kinase. Although the ultimate goal is to purify rhodopsin kinase to "near homogeneity," a retinal extract was fractionated over a DEAE cellulose column, and all eluted fractions were monitored for rhodopsin kinase activity with a kinase assay (e.g., see Figure 3). As such, Palczewski et al. demonstrate that one skilled in the art would know how to apply the same assay to any fraction of a fractionating procedure, whether the fraction contains rhodopsin kinase or not. In fact, assaying multiple fractions from the fractionating procedure for rhodopsin activity is how one can screen for a fraction that contains rhodopsin kinase to "near homogeneity."

7. Similarly, in Soubeyrand et al. (1997, J. Biol. Chem. 272: 222-227; Exhibit B), an iterative sub-fractionation approach was used to purify a Phospholipase A2 enzyme from seminal plasma. Seminal plasma was fractionated over a butyl-Sepharose column, and a desorbed fraction was applied to a Sephacryl S-300 sieving column, after which fractions were tested for Phospholipase A2 activity. Again, multiple fractions were assayed for Phospholipase A2 activity whether or not the fraction contains Phospholipase A2 (e.g., see Figure 1). The routine activity assay of multiple fractions from the fractionating procedure led to the identification of a purified fraction that contains Phospholipase A2.

8. In Ostergaard et al. (1997, J. Biol. Chem. 272: 30009-30016; Exhibit C), an iterative sub-fractionation approach was used to purify an L-Galactono-γ-Lactone Dehydrogenase (GLDase) from cauliflower plants. A crude cauliflower mitochondrial extract was passed over a DEAE-Sepharose column, and eluted fractions were assayed for GLDase by monitoring the reduction of Cytochrome *c*. Fractions having activity in this assay were passed over a phenyl-Sepharose CL-4B column, and eluted sub-fractions were assayed for GLDase activity using the same assay. The active sub-fractions were pooled and applied to a Sephacryl SF-200 gel filtration column, and the sub-fractions were monitored for activity using the same

GLDase assay. Subsequent sub-fractionation steps included two different anion exchange FPLC sub-fractionations and an HPLC sub-fractionation, with GLDase activity monitoring of the sub-fractions using the same assay at each stage. Again, multiple fractions and sub-fractions, whether containing GLDase or not, are routinely assayed for GLDase activity which led to the purification of GLDase (e.g., see Figure 1).

We hereby declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12/8/04
Date

Joel V. Weinstock, M.D.

12/8/04
Date

David E. Elliott, M.D., Ph.D.

EXHIBIT A

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Purification and Characterization of Rhodopsin Kinase*

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Rhodopsin kinase was purified to near homogeneity by affinity binding to light-exposed rod cell outer segment membranes, followed by DEAE-cellulose and hydroxyapatite chromatography. This resulted in a 1055-fold purification of highly active rhodopsin kinase with an overall recovery of 19%. Rhodopsin kinase is a single polypeptide chain with M_r = 67,000–70,000 as determined by gel filtration and SDS-PAGE.

The kinetic parameters of the enzyme for freshly bleached rhodopsin are K_m = 4 μM and V_{max} = 700 nmol/min/mg whereas for ATP K_m = 2 μM (which is a low value for kinases generally, and about 20 times lower than comparable measurements for a kinase of a similar type, the β-adrenergic-receptor kinase (Benovic, J. L., Mayor, F. Jr., Staniszewski, C., Lefkowitz, R. J., and Caron, M. G. (1987) *J. Biol. Chem.* 262, 9026–9032). GTP, on the other hand, is a very poor substrate (K_m = 1 mM, V_{max} = 10 nmol/min/mg). Rhodopsin kinase is competitively inhibited by adenosine and its mono- and diphosphate derivatives, but not by most other adenosine derivatives. Based upon measurements with 28 nucleotide derivatives, the ATP-binding site of rhodopsin kinase appears to have more specific requirements than that for other kinases.

Compounds such as cGMP, inositol trisphosphate, and others that change concentration during exposure of rod cells to light have only minor inhibitory effects on the kinase activity, with the exception of inositol monophosphate, which can activate the kinase about 20% at 50–100 μM. Rhodopsin kinase has been difficult to store with retention of activity, but can be successfully stored frozen at −20 °C in 20% adonitol.

Rhodopsin is the best characterized member of a family of homologous receptors that operate via G-proteins (the adrenergic receptors, muscarinic acetylcholine receptor, etc. (1)). As part of their functions as signal transducers, the receptors become phosphorylated by protein kinase(s) in a reaction believed to deactivate the receptor. Rhodopsin kinase is the enzyme which phosphorylates light-exposed rhodopsin in rod cell outer segments. We have purified rhodopsin kinase from retinal rod cells in order to better study its enzymatic properties and role in receptor inactivation.

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This work is dedicated to the memory of our colleague and friend, Hermann Kühn.

¶ Supported by a Jules and Doris Stein Professorship from Research to Prevent Blindness, Inc.

Rhodopsin kinase was first detected when retinal rod outer segments were incubated with [^{32}P]ATP in the presence of light, resulting in the incorporation of ^{32}P into rhodopsin (2–4). The phosphorylation of rhodopsin has since been shown to occur *in vivo* (5). The reaction has been studied in intact whole retinas (6), homogenized retinas (7), rod outer segments (8), and with rhodopsin kinase which has been submitted to one or more steps of purification (9–12). Many properties of the enzyme have been elucidated (see Ref. 13), but the kinase has not been obtained in purified form or studied in the detail available for many other kinases (14). A purification procedure for rhodopsin kinase was published recently (15). However, the specific activity of the rhodopsin kinase preparation (10 nmol of phosphate/min/mg) is considerably less than we report here (960 nmol/min/mg). Its molecular mass is a matter of disagreement, with reports ranging from M_r = 52,000 (10) to M_r = 79,000 (11). Although many workers have reported that rhodopsin kinase activity is not influenced by cyclic nucleotides (16–18), there are reports to the contrary (19–21). A number of substrate analogues have been tested for their influence on rhodopsin kinase activity, but a systematic study of the requirements of rhodopsin kinase's ATP binding site has not been undertaken. Such studies are best performed on the purified enzyme.

An enzyme which appears to be similar to rhodopsin kinase, has been shown to phosphorylate the ligand-bound form of the β- and α_2-adrenergic receptors (22, 23). This enzyme, β-adrenergic receptor kinase, phosphorylates the β-adrenergic receptor leading to homologous receptor desensitization (24, 25). Rhodopsin kinase is able to phosphorylate the β-adrenergic receptor (26), thus indicating that not only are the receptors members of a related family, but that these two kinases may be members of a kinase family specific for receptors which operate via G-proteins. A kinase of this type may also be responsible for phosphorylation of the muscarinic acetylcholine receptor (27). In this investigation we report that the purified rhodopsin kinase shares many of the properties of the β-adrenergic receptor kinase.

MATERIALS AND METHODS

Isolation of Rod Outer Segments—Rod outer segments (ROS)[1] were prepared from fresh bovine retinas obtained locally, or from frozen retinas (Lawson, Inc., Omaha, NE). We followed the procedure of Schnetkamp *et al.* (28) for the preparation of intact rod outer segments and the procedure of Wilden and Kühn (8) for preparation of membrane-bound rhodopsin to use as a substrate for rhodopsin kinase. ROS were also prepared from frozen retinas under normal room illumination but otherwise following the procedure of Wilden

[1] The abbreviations used are: ROS, rod cell outer segments; DTT, dithiothreitol; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; AMPPCP, adenosine 5′-(β,γ-methylene)triphosphate; HPLC, high performance liquid chromatography; $Mg(OAc)_2$, magnesium acetate.

and Kühn (8). These preparations yielded as much rhodopsin kinase activity as that obtained from ROS prepared from fresh retinas in dim red light by the method of Schnetkamp. ROS preparation and all kinase separation steps were carried out at 4 °C.

Extraction of Rhodopsin Kinase Activity from Rod Outer Segments—ROS from 400 retinas were suspended in 400 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM $Mg(OAc)_2$, 5 mM DTT, and 30 μg/ml each of the protease inhibitors aprotinin, benzamidine, leupeptin, and pepstatin. The suspension was homogenized with a glass-glass homogenizer and then illuminated at 0 °C for 2 h with a 150-watt floodlamp approximately 20 cm from the sample. The ROS membranes were then collected by centrifugation at 16,000 × *g* for 30 min at 4 °C. The supernatant from this centrifugation is referred to as the "Mg extract" and contains the other kinase activities found in ROS (*e.g.*, cAMP-dependent kinase and protein kinase C. We have been able to prepare protein kinase C from this extract as previously described by Kelleher and Johnson (29)). The ROS membrane pellets were then resuspended in 100 ml of 20 mM Tris-HCl (pH 7.5) containing 10 mM DTT, 60 mM KCl, and 20 μg/ml each of aprotinin, benzamidine, leupeptin, and pepstatin. The suspension was homogenized with a glass-glass homogenizer and then stirred for 12 h in the dark at 4 °C. The supernatant from this extract, referred to as the "KCl extract," was collected after centrifugation at 16,000 × *g* for 45 min at 4 °C. If kinase yields of less than 70–75% were obtained, the KCl extraction step was repeated. If required, the supernatant was recentrifuged in order to remove all ROS membranes.

DEAE-cellulose Chromatography of Rhodopsin Kinase—The pH of the above KCl extract was adjusted to 7.8 (by addition of 10% Tris solution) and the extract was diluted to a concentration of 50 mM KCl before applying to a 1.6 × 20-cm column of DEAE-cellulose (Whatman DE52) which had been equilibrated with 75 mM Tris-HCl buffer containing 1 mM $Mg(OAc)_2$ and 1 mM DTT. Fractions of 2 ml were collected from the column at a flow rate of 12 ml/h. The column was monitored at 280 and 225 nm, and for kinase activity using 50 μl of each fraction in the kinase assay described below. After loading, the column was washed with equilibrating buffer until the $A_{280\,nm}$ or $A_{225\,nm}$ returned to baseline. Then a KCl gradient (250 ml total, from 0 to 0.25 M KCl, in the same buffer) was used to elute rhodopsin kinase.

Hydroxyapatite Chromatography of Rhodopsin Kinase—A hydroxyapatite column (1.0 × 10 cm) was prepared and equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM $Mg(OAc)_2$ and 1 mM DTT. Pooled fractions containing rhodopsin kinase from the DEAE-cellulose chromatography were loaded directly onto the hydroxyapatite column, and the column was washed with equilibrating buffer at a flow rate of 10 ml/h until the $A_{220\,nm}$ returned to baseline. The column was then eluted with equilibrating buffer containing 0.4 M KCl. 2-ml fractions were collected. Aliquots (50 μl) of the fractions were used to assay for rhodopsin kinase activity as described below. Fractions containing kinase activity were pooled.

Protein Determinations—Protein concentrations were measured using the micro-Bradford method (30) with bovine serum albumin as the standard.

SDS-PAGE—Protein solutions were desalted using a Nucleosil C_4 HPLC column (32), and then SDS-PAGE was performed according to Laemmli (31) with 12% acrylamide gels in a Hoeffer minigel apparatus.

Molecular Weight Determined by Gel Filtration—Gel filtration on Sepharose CL 6B-200 was used to determine the molecular weight of the native kinase. A 1.6 × 100-cm column (Pharmacia LKB Biotechnology Inc.) was prepared using gel equilibrated with 67 mM potassium phosphate (pH 7.0) which contained 0.1 mM EDTA, 1 mM $Mg(OAc)_2$, and 5 mM DTT. The column was developed with this buffer at a flow rate of 10 ml/h, and 2.2-ml fractions were collected. Molecular weight standards used were aldolase (160,000), bovine serum albumin (67,000), ovalbumin (43,000) carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000) with blue dextran included to indicate the void volume and sodium azide to mark the included volume. For standardization, the column was monitored by absorbance at 280 or 220 nm. For molecular weight determination of rhodopsin kinase, the KCl extract from 30 retinas containing about 5 μg of rhodopsin kinase in 3 ml of extraction buffer (20 mM Tris-HCl, pH 7.5, containing 10 mM DTT and 60 mM KCl) was submitted to gel filtration on the standardized column. The column was monitored for kinase activity by withdrawing an aliquot of each fraction for the kinase assay.

Assay for Rhodopsin Kinase Activity—All rhodopsin kinase assays were performed using rhodopsin in urea-washed ROS membrane as substrate (13). Briefly, rhodopsin kinase was incubated under illumination at 25 °C with 10 μM rhodopsin, 100 μM [γ-^{32}P]ATP (1–3 × 10^4 cpm/nmol) in 75 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT and 1 mM $Mg(OAc)_2$. The reaction was stopped with 10% trichloroacetic acid solution containing 10 mM H_3PO_4. Rhodopsin-containing membranes were collected by centrifugation and the pellet was washed repeatedly with the trichloroacetic acid/H_3PO_4 solution to remove unbound ^{32}P. Concentrations of effectors were determined spectrophotometrically using extinction coefficients as described (33, 34). Inhibition constants were obtained by nonlinear regression analysis and averaging of results from at least two separate experiments. Concentrations of inhibitors (Sigma) were in the range of ~0.2–8 × K_m. All experiments for determination of K_i were performed with rhodopsin kinase prepared on a small scale (100 retinas) through the DEAE-cellulose chromatography step.

Stability of Rhodopsin Kinase—Activity of rhodopsin kinase was determined under standard conditions following freezing (in liquid nitrogen or at −20 °C) or exposure to defined temperatures for 2 h. The effect of the following polyalcohols or carbohydrates was examined: adonitol, sucrose, α-methylmannopyranose, *myo*-inositol, sorbitol, erythritol, mannose, maltose, glucose, fructose, and glycerol. Compounds were used at a concentration of 20%, except for *myo*-inositol which was used at 90% of saturation.

RESULTS

Molecular Weight of Rhodopsin Kinase by Gel Filtration—The molecular weight of the protein kinase which phosphorylates bleached rhodopsin was determined as shown in Fig. 1. We find a major peak with M_r = 67,000 (in agreement with an earlier report (35)). This is consistent with the molecular weight observed on SDS-PAGE after purification of the kinase to homogeneity (M_r = 70,000 protein band). This indicates that the kinase is composed of a single polypeptide chain and contains no subunit structure. A minor peak of activity was observed eluting at a molecular weight of about 50,000. This component makes up about 10% of the activity in this preparation (and about 5% in another preparation which was examined). This minor component may be a product of proteolysis of the 67,000 protein (see "Discussion").

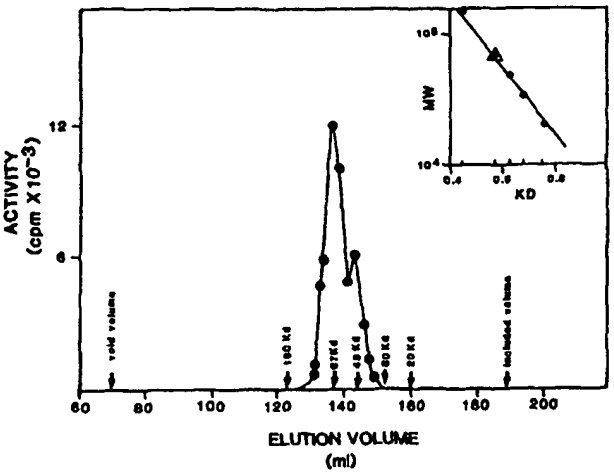

FIG. 1. **Determination of the molecular weight of rhodopsin kinase by gel filtration.** Rhodopsin kinase, partially purified by affinity binding to bleached ROS membranes, was submitted to gel filtration on a standardized Sepharose CL-6B column as described under "Materials and Methods." Elution positions of the molecular weight standards are indicated by *arrows*. Kinase activity was monitored by assaying aliquots of each fraction for ability to phosphorylate bleached ROS membranes. The counts incorporated into rhodopsin in this assay are plotted *versus* elution volume. In the *inset*, log (*MW*) of the standards is plotted *versus* kilodalton (*KD*) with the *open triangle* indicating the molecular weight of the major peak of kinase activity at 67,000. A minor peak of activity is observed at 50,000. The same results were obtained in duplicate experiments.

TABLE I

Purification of rhodopsin kinase

Rhodopsin kinase was purified as described under "Materials and Methods." The standard procedure used 400 retinas. Protein concentration was determined by the micro-Bradform method (30). Kinase activity was measured at 25 °C using urea-washed rod outer segments and [γ-^{32}P]ATP as substrates. The table summarizes results from nine preparations.

Purification step	Volume	Protein	Activity	Specific activity	Purification		Yield	
					Step	Overall	Step	Overall
	ml	*mg*	*nmol P_i/min*	*nmol P_i/min/mg protein*	*-fold*		*%*	
Rod outer segments	400	280 ± 28	255 ± 32	0.91	1	1	100	100
Rod outer segments after Mg extract	110	231 ± 18	264 ± 40	1.14	1.25	1.15	103	103
Mg extract	380	52 ± 11	18 ± 6	0.35				
KCl extract	100	22 ± 5	168 ± 60	7.6	83	83	66	66
DE-cellulose 52	50	1.5 ± 1	92 ± 40	61	8	100	54	36
Hydroxyapatite	15	0.05 ± 0.016	48 ± 11	960	15	1055	52	19

Purification of Rhodopsin Kinase—The first step in obtaining a pure rhodopsin kinase preparation is to extract the kinase from the retina or outer segments. We found that when rhodopsin kinase was prepared from sealed ROS made by the Schnetkamp method (28), or from ROS prepared from retinas under room lights, approximately twice the yield of kinase was obtained compared with that from ROS prepared by standard methods. The extraction step of our kinase preparation was based on the protocol of Sitaramayya (12) with several modifications which include shorter times for the extractions. More Mg^{2+} (10 mM) was included in the Mg^{2+} extracting buffer in order to improve binding of kinase to rhodopsin. Salt concentration was reduced in the KCl extraction buffer in order to achieve better binding of kinase to the ion exchange resin in the next purification step. The KCl extraction was performed in the dark in order to facilitate dissociation of the light-induced binding of the rhodopsin kinase to rhodopsin. Essentially all of the rhodopsin kinase activity remains with the membrane pellet when 10 mM Mg^{2+} is used (Table I), whereas in the presence of 1 mM Mg^{2+}, from 20–40% activity was lost at this step. This extraction step also appears to achieve a separation of rhodopsin kinase from the other protein kinase activities in the ROS. Extraction of the pellet with approximately isomotic KCl-Tris buffer in the dark yielded about 70% of rhodopsin kinase activity in the supernatant.[2]

By using DEAE-cellulose (Whatman DE52), kinase was bound to the resin and eluted at low ionic strength with a yield of 54% (Fig. 2 and Table I). DEAE-Sephacel (Pharmacia) can be substituted for DE52 (Whatman), but no rhodopsin kinase activity could be recovered from DEAE-cellulose (Sigma).

When the rhodopsin kinase peak was pooled and loaded on the hydroxyapatite column, the rhodopsin kinase activity was bound to the column and 52% was recovered upon elution using a step gradient (Fig. 3 and Table I). SDS-PAGE indicated the presence of essentially one protein with a M_r = 70,000 in the rhodopsin kinase activity pool from this hydroxyapatite column. The hydroxyapatite column gave much better resolution of rhodopsin kinase using a gradient with KCl rather than with potassium phosphate. This behavior is typical for a basic protein (36).

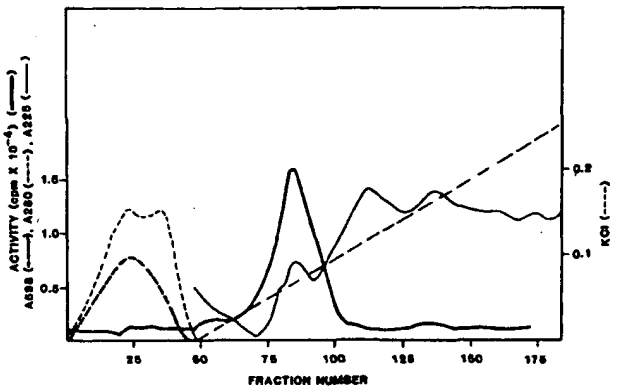

FIG. 2. **DEAE-cellulose chromatography of rhodopsin kinase.** The KCl extract from 400 bovine retinas was adjusted to pH 7.8 and then applied to the DEAE-cellulose column (1.6 × 20 cm) equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM DTT and 1 mM $Mg(OAc)_2$. The column was washed until the absorbance of the effluent at 280 nm fell below 0.05. The elution was carried out with a linear KCl gradient (2 × 125 ml of 0–0.25 M) in the same buffer. 2.0-ml fractions were collected. The protein content was determined by the micro-Bradford method (30).

Stability of Rhodopsin Kinase—It has proven difficult to store rhodopsin kinase with retention of activity. Shichi and Somers (10) reported that at 3 °C, 50% of the activity was lost in a week. They found that K^+, NH_4^+, and glycerol stabilized the activity somewhat. Many investigators have found that crude extracts lost their activity in a few days when stored in the cold. Sitaramayya (12) found that without protease inhibitors, 90% of the activity was lost in 4–5 days whereas in the presence of protease inhibitors, 76% of the original activity remained after 37 days. We find that the half-life of our KCl extract is about 30 days when stored at 4 °C. The DE-cellulose preparation of rhodopsin kinase has a half-life of about 7–15 days, and highly purified kinase has a half-life of about 3–5 days. Dilution of the purified rhodopsin kinase is also deleterious to kinase activity speeding the decay of the activity. In a similar system, Benovic *et al.* (22) observed a half-life of the β-adrenergic receptor kinase of 5–10 days. In addition, we have observed a very marked sensitivity of rhodopsin kinase to organic solvents (*e.g.* an incubation of only 2 or 3 min with 5% ethanol reduces the activity by 45%).

In order to facilitate experimentation with rhodopsin kinase, we sought conditions under which it could be conveniently frozen, incubated, and assayed with retention of activity. Kinase samples lost 80% activity when frozen at liquid

[2] Several different chromatographic separations were tried in our attempts to purify rhodopsin kinase from the KCl extract, in addition to the successful approach adopted. Chromatography on CM-cellulose (Sigma) using 75 mM Tris-HCl, 1 mM $Mg(OAc)_2$, 1 mM DTT (pH 7.8) yielded a recovery of only 20% of the loaded activity, and this activity did not bind to the column. Changing the pH to 6.8 resulted in a complete loss in recoverable activity. Similarly, chromatography on phosphocellulose (Sigma) in this buffer at pH 7.5 yielded no recoverable activity.

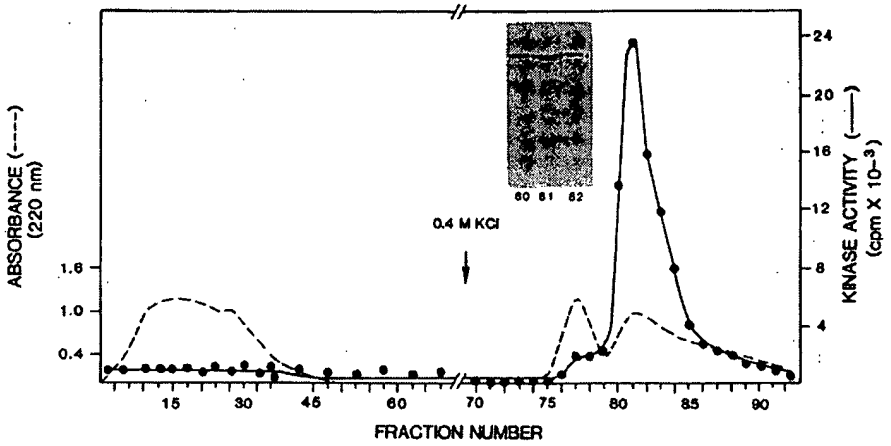

FIG. 3. **Hydroxyapatite chromatography of rhodopsin kinase.** The combined fractions containing rhodopsin kinase eluted from DEAE-cellulose (Fig. 2) were loaded on a hydroxyapatite column (1.0 × 10 cm) equilibrated with 75 mM Tris-HCl buffer (pH 7.8) containing 1 mM dithioerythritol and 1 mM $Mg(OAc)_2$. Then the column was washed with the same buffer, until the optical density at 220 of the effluent dropped below 0.05. The rhodopsin kinase fractions were then eluted with the same buffer containing 0.4 M KCl. 2-ml fractions were collected. All samples of kinase were desalted using reverse-phase chromatography as described under "Materials and Methods," before SDS-PAGE was performed using the Laemmli system (31) with 12% acrylamide gels. The *inset* shows stained gels of aliquots from fractions 80, 81, and 82 from the hydroxyapatite column which contain rhodopsin kinase activity.

nitrogen temperatures in water or inositol, and 30% in sucrose. No loss of activity was observed when kinase was frozen in adonitol. Similar losses of activity occurred when samples were frozen at −20 °C, except when adonitol was present, in which case no loss of activity occurred. The enzyme is stable for 2 h at 4 °C in adonitol and other solutions. Neither adonitol nor sucrose protect from activity loss at 30 °C, since 50% activity is lost after 2 h in water or the sugars. Solid sucrose and adonitol were added to kinase solutions which were then frozen at −20 °C and assayed at monthly intervals. In sucrose the enzyme lost an initial 30% activity and was stable to further storage. In adonitol there was no loss of activity over a period of months. We now routinely store our kinase frozen at −20 °C in 20% adonitol.

Effect of Cyclic Nucleotides and Inositol Phosphates on Rhodopsin Kinase Activity—Several compounds which change concentrations upon illumination of rod outer segments were tested for their effect on rhodopsin kinase activity (Fig. 4). cGMP affects rhodopsin kinase activity by less than 10% in the micromolar to millimolar concentration range. Similarly, cAMP and inositol 1,4,5-trisphosphate have no effect at concentrations below 0.1 mM. At 1 mM, these two compounds inhibited the kinase activity by about 20–30%. In contrast, D-*myo*-inositol-1-phosphate appears to stimulate rhodopsin kinase activity by about 20% at concentrations between 0.3 and 100 μM while it inhibits somewhat at higher concentrations.

Effects of Adenosine Analogues on Rhodopsin Kinase Activity—By using purified rhodopsin kinase, the K_m for rhodopsin was found to be 4 μM (Table II), a value equivalent to that reported earlier for a crude kinase preparation (13). Since Mg^{2+} has been found to affect kinase activity (13), the K_m and V_{max} for ATP were measured at two Mg^{2+} concentrations (Table II). As observed previously, the effect of high concentrations of Mg^{2+} is to inhibit the kinase, in the present case by increasing the K_m as well as by slightly reducing the V_{max}.

A variety of analogues of ATP were tested for their effect on the kinase activity (Table II). Adenosine, for example, competes with ATP with a K_i of 4 μM, a value equivalent to that of the K_m of ATP (Fig. 5). Similarly, AMP and ADP

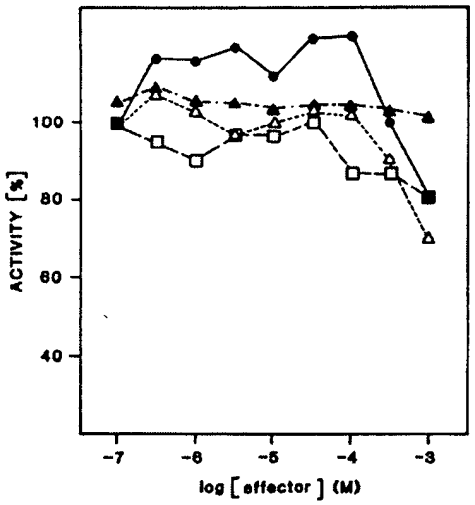

FIG. 4. **Influence of cyclic nucleotides and inositol derivatives on rhodopsin kinase activity.** The activity of rhodopsin kinase is plotted as a function of the concentration of the effector where 100% is the activity of rhodopsin kinase with no added effectors. ●, D-*myo*-inositol-1-monophosphate; □, D-*myo*-inositol-1,4,5-trisphosphate; △, cAMP; ▲, cGMP.

compete with almost equivalent K_i values. Substitution of sulfate for the phosphate of AMP reduces the inhibitory effect, but it remains a good inhibitor. Other substitutions in the 5′ position produce only poor inhibitors. Removing the hydroxyl in the 2′ position of the ribose moiety (2′-deoxyadenosine) abolishes its inhibitory effects, while removing the 3′-hydroxyl-(3′-deoxyadenosine) has little effect. Removing both hydroxyl groups (2′,3′-dideoxyadenosine) yields a good inhibitor although the K_i is increased somewhat. Other modifications tested yield only poor inhibitors. The analogues tested with modifications in the purine moiety of adenosine were also poor inhibitors of rhodopsin kinase activity. Dinucleotides have no effect on the kinase activity.

TABLE II

Effect of changes in the adenosine moiety on the K_m and V_{max} of substrates and inhibitors of rhodopsin kinase

Substrate	K_m	V_{max}	V_{max}/K_m[a]
	μM	*nmol/min/mg*	$s^{-1}\ M^{-1} \times 10^{-6}$
Rhodopsin (washed by 5 M urea)	4 ± 1.1	700	0.2
ATP at 1 mM Mg^{2+}	1.6 ± 0.7	638	0.46
ATP at 10 mM Mg^{2+}	5.2 ± 0.5	608	0.14
GTP at 2 mM Mg^{2+}	1000 ± 120	10	0.00001
Inhibitors			K_i
			μM
Adenosine			4 ± 0.4
Analogues, ribose 5′ position			
AMP			5 ± 1.1
ADP			12 ± 0.7
5′-Deoxyadenosine			10 ± 0.2
S-Adenosyl-L-methionine			>1000
CoA			>5000
Methylene adenosine 5′-triphosphate			>1000
Imidoadenosine 5′-triphosphate			>1000
Adenosine 5′-monosulfate			80 ± 2.1
2′ or 3′ position			
2′-Deoxyadenosine			No effect
3′-Deoxyadenosine			16 ± 2.1
Adenosine 2′-deoxy-3:5-monophosphate			No effect
Adenosine 2′:3′-monosphosphate			No effect
Adenosine 3′:5′-monophosphate			>3000
Adenosine 9-arabinofuranoside 5′-monophosphate			>3000
2′,3′-Dideoxyadenosine			65
Adenosine 5′-monophosphate (periodate oxidized/borohydride reduced)			>1000
Analogues, purine			
GMP			>5000
Guanosine			No effect
GDP			No effect
Xantosine 5′-monophosphate, hypoxanthine 9-arabinofuranoside			No effect
Inosine monophosphate			No effect
1-N^6-Ethenoadenosine monophosphate			No effect
Adenosine N^1-oxide			>5000
Dinucleotides			
NAD, NADH			No effect
NADP, NADPH			No effect

[a] V_{max}/K_m were calculated assuming M_r of rhodopsin kinase equal 70,000.

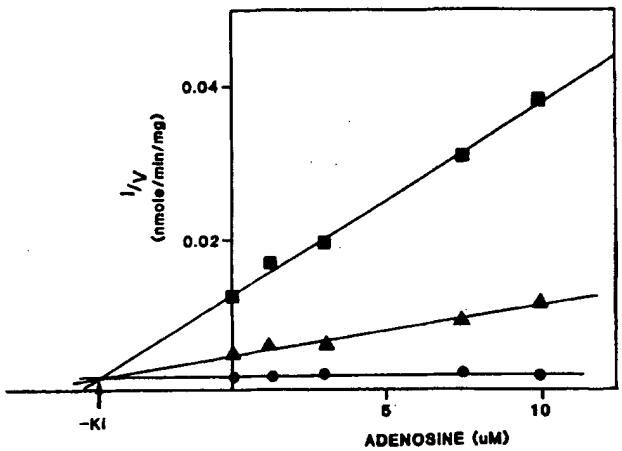

FIG. 5. **The effect of adenosine on rhodopsin kinase activity.** Rhodopsin kinase was assayed as described under "Materials and Methods" with different concentrations of adenosine (as indicated in figure). Concentrations of [γ-^{32}P]ATP used were 16.7 μM (●), 3.3 μM (▲), and 1 μM (■).

DISCUSSION

Molecular Properties of Rhodopsin Kinase—Rhodopsin kinase behaves as a soluble protein which can be extracted by either low or high ionic strength buffers. Elution of rhodopsin kinase from a C_4 HPLC column required 50% isopropanol, whereas bovine serum albumin, a protein with a similar molecular weight, requires only 30% isopropanol. This is consistent with the amino acid analysis of the purified rhodopsin kinase preparation (data not shown) and this suggests that rhodopsin kinase is more hydrophobic than bovine serum albumin.

We find the molecular weight of purified rhodopsin kinase to be $M_r \approx 70{,}000$ by SDS-PAGE. While several studies have included molecular weight determination of rhodopsin kinase by gel filtration (10, 11, 35, 37), these results have ranged from 52,000 (10) to 79,000 (11). We reexamined the molecular weight by gel filtration using a system in which the kinase activity elutes in the middle of the separation range of the system. We find a molecular weight of 67,000 which is consistent with the 69,000 observed by Kühn (35) and 68,000 observed by Lee *et al.* (37). Since the molecular weights observed by SDS-PAGE and gel filtration are the same, it is clear that no subunit structure is needed for rhodopsin kinase activity. We observed only a minor peak eluting around 50,000. This may represent the kinase activity reported by Shichi and Somers (10) and for which there is also some evidence in the gel filtration profile of Lee *et al.* (37). No significant quantities of this component are found when inhibitors of proteolysis are employed. We suspect that this $M_r = 50{,}000$ protein, which shows rhodopsin kinase activity, is a proteolytically modified form of rhodopsin kinase.

Effect of Cyclic Nucleotides and Inositol Phosphates on Rhodopsin Kinase Activity—Results from previous studies of the effect of cyclic nucleotides have been variable. Swarup and Garbers (19) found stimulation of phosphorylation of rhodopsin by cGMP at >5% bleach while at 1% bleach, a small inhibition of rhodopsin phosphorylation was observed. Shuster and Farber (20) observed a marked decrease in phosphorylation of rhodopsin in the presence of cGMP, but cAMP had almost no effect. A variety of other compounds have been examined for their effect on the phosphorylation of rhodopsin. However, since most of these studies utilized either whole ROS or relatively crude extracts containing rhodopsin kinase activity, we have reinvestigated the effects of these compounds using our assay system employing purified rhodopsin kinase and urea-washed ROS membranes. The marked effect of light on the metabolism of polyphosphoinositides in ROS (38) prompted us to examine the effect of two phosphoinositols as well. In this system, there are no other soluble enzymes that can interfere with either the kinase, the rhodopsin, or the components being tested for their effect on rhodopsin kinase. We found only a small effect of any of the compounds tested on rhodopsin kinase activity. We conclude that these compounds, *i.e.*, cAMP, cGMP, D-*myo*-inositol-1-monophosphate and D-*myo*-1,4,5-trisphosphate have no direct role in regulating rhodopsin kinase activity in the outer segment; however, they could have an indirect role by regulating other systems in the outer segments.

Effects of ATP Analogues on Rhodopsin Kinase—In this study, naturally occurring ATP analogues were employed to study rhodopsin kinase in order to determine the specificity of the ATP binding site of the rhodopsin kinase, and in order to evaluate what features of the nucleotide would be important

for future studies with covalent inhibitors. We also wanted to compare the effects of these analogues on rhodopsin kinase to their effects on other protein kinases. We have identified analogues which can block kinase activity in whole ROS as well as in a highly purified reconstituted system. The kinetic parameters are essentially the same for the purified kinase as for the kinase extract used previously (13) in which only about 10% of the protein in the extract was rhodopsin kinase. This indicates that results obtained earlier are valid and were not affected by interference from other components in the extract. Similar results were observed for the M_r = 52,000 kinase isolated by Schichi and Somers (10).

Protein kinases vary in their preference for high energy phosphate substrates. Some use ATP much more effectively than GTP (cAMP- and cGMP-dependent protein kinases, protein kinase C, hormone-stimulated ribosomal protein S6 kinase) (39–43). Others prefer GTP to ATP (growth-associated histone H1 kinase, K_m = 58 μM for ATP and 1.4 μM for GTP) (44). The K_m values for the high energy phosphate vary from 3.1 μM for protein kinase A up to 380 μM for phosphorylase kinase (45, 46). Rhodopsin kinase strongly prefers ATP as a substrate yielding a turnover for ATP about 40,000 times that of GTP (as determined from the ratio of their respective K_m values). This high affinity for ATP is based on the interaction between the nucleotide and the enzyme rather than the polyphosphate binding to the enzyme. This is indicated by the strong preference for ATP as compared to GTP as well as by the ability of adenosine to inhibit the kinase competitively with a K_i similar to the K_m of the enzyme.

An earlier study showed that rhodopsin kinase is inhibited by adenosine (10). We reinvestigated the effect of adenosine on rhodopsin kinase in order to determine the nature of this inhibition. The data presented in Fig. 5 demonstrate that adenosine is a pure competitive inhibitor with respect to ATP with a K_i of 4 μM similar to that of the K_m for the kinase for ATP. Other analogues of ATP were examined for their effect on rhodopsin kinase in order to give us information about the ATP binding pocket of rhodopsin kinase. As shown in Table II, modification of the 5′ position of adenosine by phosphorylation, sulfonation, or removal of the hydroxyl group has little effect on the K_i (*e.g.* K_i for AMP is 5 μM); see "Results" for AMP, ADP, adenosine 5′-monosulfate, 5′-deoxyadenosine). However, substitutions like *S*-adenosyl-L-methionine, dinucleotide (NAD^+ or NADH) or CoA dramatically change the K_i for rhodopsin kinase probably due to steric interactions. These data are quite different from those reported for cAMP- and cGMP-dependent protein kinase where changing the charge in the 5′ position (via phosphorylation) has a major effect on the interaction between enzyme and nucleotide (47). For this reason, the K_i values for adenosine for cAMP- and cGMP-dependent protein kinase are 0.54 and 1.7 mM, respectively, much higher than the K_m for ATP. On the other hand, the K_i of adenosine for calmodulin-dependent protein kinase is lower (250 μM (48)). For rhodopsin kinase, the charge does not play a big role, although some modifications in this region disrupt the binding to the kinase. For example, the nonhydrolyzable analogs AMPPNP and AMPPCP have lost most of their inhibitory effect for rhodopsin kinase while for other kinases they are effective inhibitors.

For cAMP-dependent protein kinase, cGMP-dependent protein kinase, and calmodulin-dependent protein kinase, the 3′-deoxy derivative of ATP has an even higher affinity for the kinase than ATP while the 2′-deoxy derivative has a lower affinity (47, 48). For rhodopsin kinase, these same modifications yield similar but not identical effects. The 3′-deoxy derivative binds almost as well as ATP while the 2′-deoxy derivative has completely lost its ability to bind. The 2′,3′-dideoxy derivative is a fairly good inhibitor whereas substituting a ribose isomer, adenosine 9-arabinofuranoside-5′-monophosphate again destroys the ability to inhibit. Another analogue is missing the 2′-3′ carbon-carbon bond, but retains the hydroxyl groups (adenosine 5′-monophosphate (periodate oxidized borohydride reduced)) and this compound also has little inhibitory effect. From these data, it is not clear whether the critical component for binding to the kinase is the hydroxyl group at position 2′ or the conformation of the ribose moiety or some combination of these two. It is possible that there is a carboxylic residue around the 2′ position in the active site of rhodopsin kinase similar to enzymes which have a Rossmann domain (49) because the presence of phosphate causes a loss in affinity for the enzyme (*e.g.*, 3′,5′-cAMP *versus* 2′,3′-cAMP).

Examining another region of the ATP molecule, we tested a few analogues with changes in the purine moiety. None of these analogues was an effective inhibitor of rhodopsin kinase, suggesting that the adenine moiety itself is very specifically recognized by rhodopsin kinase and not only by simply the appropriate conformation around the *N*-glycosylated bond. To summarize, it appears that rhodopsin kinase's ATP domain has similarities to those of cAMP- and cGMP-dependent protein kinase and calmodulin-dependent protein kinase, although it appears that the ribose moiety must be in the proper conformation for binding. It is also likely that the triphosphate chain has some special conformation acceptable in the active site of rhodopsin kinase since some substitutions yield poor inhibitors. The ATP-binding requirements of rhodopsin kinase therefore appear to be more specific than that of other kinases.

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EXHIBIT B

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Purification of a Novel Phospholipase A_2 from Bovine Seminal Plasma*

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Phospholipases A_2 are enzymes believed to play important roles in numerous physiological systems including sperm cell maturation. Relatively little work has, however, been devoted to study these enzymes in seminal plasma. We therefore undertook the purification and characterization of this enzyme from bovine seminal plasma. After a 330-fold purification, an activity corresponding to a protein of 100 kDa was identified by gel filtration. SDS-polyacrylamide gel electrophoresis analysis of the purified fraction revealed the presence of a 60-kDa band that comigrated with the activity during ion-exchange and gel filtration chromatography as well as polyacrylamide gel electrophoresis. The enzyme possessed a pH optimum around pH 6.5 and was calcium-dependent. Using isoelectric focusing, its isoelectric point was determined to be 5.6 ± 0.07. The enzymatic activity was resistant to *p*-bromophenacyl bromide, but was sensitive to gossypol and dithiothreitol. The enzyme was 2 orders of magnitude more active toward micelles formed with deoxycholate than with Triton X-100. Slight differences in the specificity toward head groups and/or *sn*-2-side chains were found in both assay systems. The enzyme was acid-labile and did not display affinity for heparin. It would therefore appear that the phospholipase A_2 form isolated from bovine seminal plasma is of a novel type.

Phospholipases A_2 (PLA_2)[1] are ubiquitous enzymes capable of hydrolyzing the *sn*-2-position of phospholipids. Most PLA_2 characterized to date belong to either one of two main groups: high and low molecular mass PLA_2 (1, 2). High molecular mass PLA_2, also called cytoplasmic PLA_2 ($cPLA_2$), are 85-kDa proteins found in the cytoplasm of several cell types (3–6). They are specific for arachidonic acid (6) and possess limited lysophospholipase (7, 8) and phospholipase A_1 (9) activities. Low molecular mass PLA_2 ($sPLA_2$) form a family of homologous enzymes with molecular masses ranging from 14 to 20 kDa that are found in several secretory fluids as well as in the cytoplasm of various cell types (1, 2, 10). PLA_2 are believed to be important regulatory enzymes in numerous physiological systems such as inflammation, membrane remodeling, and cell signalization (11). Several PLA_2 that do not belong to either category have also been identified in various tissues and organisms (12–19).

In the reproductive system, PLA_2 are widely accepted to play a major role in the late maturational events of spermatozoa, particularly in the acrosomal reaction (20–23). The acrosomal reaction is a multifusion process that permits the release of hydrolytic enzymes, which are required for spermatozoa to penetrate the acellular layers surrounding the oocyte (24).

Although several studies have been undertaken to characterize the PLA_2 present in the spermatozoa and seminal plasma of various species (25–30), only the enzyme from human seminal plasma has been purified to homogeneity and sequenced (31) so as to conclusively assign it to a particular PLA_2 group. The enzyme was found to be a 14-kDa protein, identical to the synovial enzyme (32), suggesting the same might be true of other mammalian species.

In bovine seminal secretions, the enzyme was partially purified, but was not characterized enough to assign it to a particular PLA_2 group (30). To determine the exact type(s) of PLA_2 present in bovine seminal plasma and to assess the generality of the occurrence of $sPLA_2$ in mammalian seminal plasma, we purified and characterized the major PLA_2 activity from bovine seminal plasma.

EXPERIMENTAL PROCEDURES

Materials

Sephacryl S-300, butyl-Sepharose Fast Flow, and Q-Sepharose Fast Flow were purchased from Pharmacia Biotech (Baie d'Urfée, Québec, Canada). Electrophoresis reagents (including ampholytes) were obtained from Bio-Rad. Heparin, gossypol, and *p*-bromophenacyl bromide were from Sigma. Phosphatidylcholine (PC) (L-α-1-palmitoyl-2-[^{14}C]linoleoyl (specific activity of 55.6 mCi/mmol) and L-α-1-palmitoyl-2-[^{14}C]arachidonyl (specific activity of 52.6 mCi/mmol)) and phosphatidylethanolamine (PE) (L-α-1-palmitoyl-2-[^{14}C]arachidonyl (specific activity of 55.6 mCi/mmol)) were obtained from New England Nuclear (Mississauga, Ontario, Canada). The scintillation fluid (Universol) was purchased from ICN (Montreal). Aluminum-backed silica gel TLC plates were from Whatman (Maidstone, United Kingdom). Recombinant PLA_2 (porcine pancreatic and *Crotalus atrox*) were from Sigma. Dialysis membranes were from Spectrum Medical Industries, Inc. (Houston, TX). Ultrafiltration membranes were from Amicon, Inc. (Beverly, MA). All other chemicals used were of analytical grade and were purchased from commercial suppliers. Bovine semen was a generous gift from the Centre d'Insémination Artificiel du Québec (St.-Hyacinthe, Québec, Canada).

Phospholipase A_2 Assay

Enzymatic activity was assayed using *sn*-2-radiolabeled 2-arachidonyl-PE unless specified otherwise. The substrate (20,000 cpm/tube, 1.7 μM) was evaporated under nitrogen and resuspended in buffer A (50 mM

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[1] The abbreviations used are: PLA_2, phospholipase(s) A_2; $cPLA_2$ cytoplasmic phospholipase(s) A_2; $sPLA_2$, low molecular mass phospholipase(s) A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; pBPB, *p*-bromophenacyl bromide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

Tris-HCl, 0.02% NaN_3, pH 7.4) containing 10 mM sodium deoxycholate. The substrate solution was vortexed and mixed for 20 min. Ten μl of substrate solution was added to each assay tube. A typical reaction mixture (final volume of 100 μl) consisted of 1 mM $CaCl_2$ and 1 mM sodium deoxycholate in buffer A. After 30 min at 37 °C, the reaction was stopped by adding 200 μl of chloroform/methanol (2:1) containing 2 μg/ml fatty acid tracer and 50 μl of 4 M KCl. The assay tubes were then centrifuged, and the lower phase was applied onto a silica TLC plate, which was then developed in petroleum ether/ether/acetic acid (85:15:1). The fatty acids were visualized with iodine, and the stained spots were cut into scintillation vials. The scintillation fluid was then added, and the radioactivity was determined in a liquid scintillation counter.

Purification Methods

Seminal Plasma Preparation—Pools of bovine ejaculates were centrifuged at low speed (300 × *g*) to remove spermatozoa. The supernatant was then preserved at −20 °C and used for purification within 2 weeks.

Butyl-Sepharose Chromatography—Ten ml of frozen seminal plasma was thawed, adjusted to 0.1 M choline chloride, and centrifuged at 10,000 × *g*, and the supernatant was loaded (2 ml/min) on a 2.5 × 10-cm butyl-Sepharose column equilibrated in buffer A containing 0.1 M choline chloride. The column was then washed at 7 ml/min with 700 ml of equilibration buffer followed by 350 ml of 5 M urea in buffer A (Fraction I).

Sephacryl S-300 Chromatography—Fraction I was concentrated by ultrafiltration (pore size of 10,000; Amicon, Inc.) and applied to a 1.5 × 110-cm Sephacryl S-300 column (4 °C) equilibrated in buffer A containing 0.15 M choline and 0.15 M NaCl. Fractions (5.8 ml) were collected at a flow rate of 0.3 ml/min. The fractions under the activity peak were pooled and concentrated (Fraction II). Calibration of the column was performed under the same conditions by passing RNase A, ovalbumin, and bovine serum albumin.

Q-Sepharose Chromatography—Fraction II was applied to a Q-Sepharose column (1 × 1 cm) coupled to a fast protein liquid chromatography system and equilibrated in buffer A (without NaN_3) containing 0.2 M NaCl. The active fractions were eluted with a 0.2–1 M NaCl gradient in buffer A. Fractions (1 ml) were collected at a flow rate of 1 ml/min.

Characterization

A partially purified (190-fold) fraction, obtained by an alternative lower yield approach, was preserved at −20 °C in 25% glycerol and used for all characterization studies unless otherwise specified. The substrate used was arachidonyl-PE unless specified otherwise.

pH Dependence—The following buffers were used for pH dependence studies: pH 4–5, 50 mM sodium acetate; pH 6.5–7, 50 mM MES; pH 7.5–8.5, 50 mM Tris-HCl; pH 9–10.5, 50 mM ethanolamine; and pH 11–11.5, 50 mM CAPS. The reaction was carried out at 22 °C.

Isoelectric Focusing—Isoelectric focusing was performed at 22 °C for 7000 V-h on a post Sephacryl S-300 aliquot adjusted to 5 M urea and 2% ampholytes. The gel rods (0.3 × 13 cm) consisted of 4% acrylamide, 2% ampholytes, pH 3–10, 2% Triton X-100, and 5 M urea. After completion of the electrophoresis, the gel rod was cut into 24 pieces, and proteins were eluted in 500 μl of H_2O/piece at 4 °C for 16 h on an orbital shaker.

Inhibition Studies—For inhibition studies, PLA_2-containing fractions were preincubated with the indicated concentrations of inhibitor dissolved in dimethyl sulfoxide (pBPB and gossypol) or H_2O (dithiothreitol (DTT)) for 3 h (pBPB) or for 30 min (gossypol or DTT) at 37 °C in buffer A. The sample was diluted 10 times prior to the enzymatic assay so that the final concentration of dimethyl sulfoxide in the assay tube was 1%.

Protein Estimation—During purification, protein concentration in each fraction was estimated by monitoring the absorbance at 280 nm. Protein content in pooled fractions was determined according to Bradford (33).

SDS-PAGE and Related Techniques—SDS-polyacrylamide gel electrophoresis (PAGE) was performed essentially as described by Laemmli (34). PAGE was performed on a 6% gel according to Kramer *et al.* (35). The apparent molecular mass of the various protein bands was determined with the low molecular mass calibration kit from Pharmacia Biotech. Proteins were visualized using Coomassie Brilliant Blue R-250 (36).

RESULTS

Purification of Bovine Seminal PLA_2

Seminal plasma was first passed through a butyl-Sepharose resin (Fig. 1*a*). Extensive washing (14 column volumes) was required to remove all the weakly adsorbed proteins. The urea-

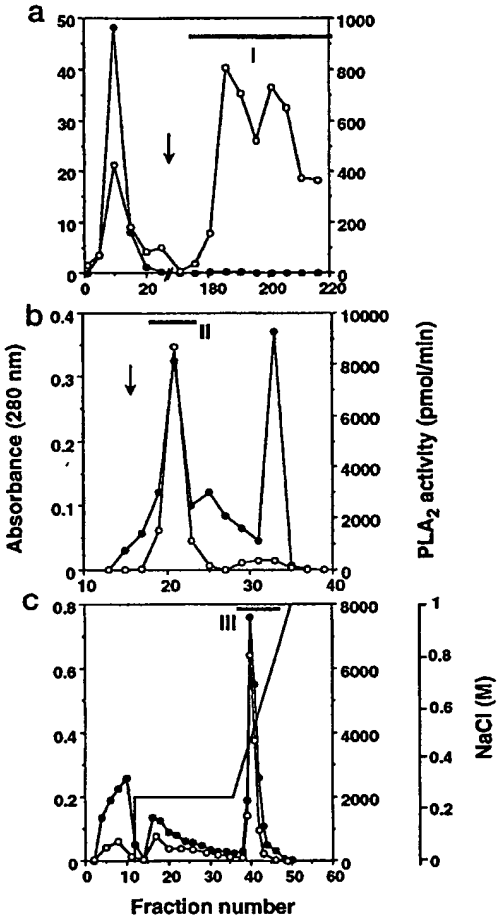

FIG. 1. **Purification scheme.** *a*, butyl-Sepharose chromatographic pattern; *b*, Sephacryl S-300 chromatographic pattern; *c*, Q-Sepharose chromatographic pattern. Chromatography was performed as described under "Experimental Procedures." The fractions under the *bar* were pooled and assayed for purity as indicated in Table I. Where appropriate, the gradients used are indicated. The *arrow* in *a* indicates the point of addition of the 5 M urea buffer. In *b*, the approximate void volume of the Sephacryl S-300 column is indicated by the *arrow*. ●, absorbance; ○, PLA_2 activity.

TABLE I
Purification summary

Aliquots of the various fractions obtained during purification were assayed for activity and protein content as described under "Experimental Procedures." One activity unit corresponds to 1 pmol of PE hydrolyzed per min.

Step	Activity	Protein	Specific activity	Yield	Purification
	units	*mg*	*units/mg*	%	*-fold*
Seminal plasma	24,000	730	33	100	1
Butyl-Sepharose FF (I)	28,000	6.6	4,200	120	130
Sephacryl S-300 (II)	32,000	3.9	8,200	130	250
Q-Sepharose FF (III)	11,000	1.0	11,000	45	330

FIG. 2. **SDS-PAGE of active pools obtained during purification.** The pooled fractions (~5 μg of protein/pool) were subjected to SDS-PAGE. *Lane 1*, 3 μg of molecular mass markers (LMW, Pharmacia Biotech); *lane 2*, seminal plasma; *lane 3*, Fraction I; *lane 4*, Fraction II; *lane 5*, Fraction III. The samples were adjusted with reducing SDS-PAGE buffer, boiled for 10 min, and loaded onto a 12% SDS-polyacrylamide Mini-Gel (Bio-Rad), which was then stained with Coomassie Brilliant Blue. The migration of the molecular mass markers corresponds (from top to bottom) to 94, 67, 43, 30, 20.1, and 14.4 kDa, respectively.

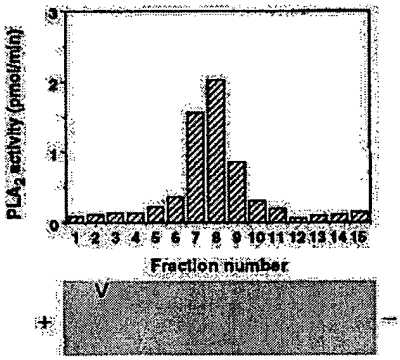

FIG. 3. **Native PAGE of purified PLA_2.** Ten μg of purified enzyme was loaded in each of two lanes of a polyacrylamide gel. After electrophoresis (100 min, 200 V), one lane was cut into 15 pieces, which were then eluted for 20 h (4 °C) in 500 μl of 0.1 M NH_4HCO_3, while the other lane was stained with Coomassie Brilliant Blue. The eluate of each fraction was assayed for PLA_2 activity. The *V* indicates the migration position of bovine serum albumin under the same conditions.

desorbed fractions (Fraction I) contained most of the recovered activity. Fraction I was concentrated and loaded onto a Sephacryl S-300 gel sieving column (Fig. 1*b*). A single active peak was obtained whose elution position corresponded to the behavior of a 100-kDa protein as determined by calibration of the column. The active peak was then concentrated and applied onto a Q-Sepharose ion exchanger. The activity was again eluted in one major activity peak, which well overlapped the protein pattern (Fraction III).

The purification results are summarized in Table I. This scheme resulted in a purification of 330-fold with a 45% recovery of the activity.

Characterization of Bovine Seminal PLA_2

The Purified Enzyme Behaves as a 60-kDa Protein on SDS-PAGE—The active fractions from the successive purification steps were analyzed by SDS-PAGE (Fig. 2) under reducing conditions. After a single purification step (Fraction I; *lane 3*), a main component at 60 kDa is visible. This component then persists throughout until the end of the purification procedure, where it is the only major band detectable by Coomassie Blue staining (Fraction III; *lane 5*).

The 60-kDa Band Is Responsible for the Activity—Fraction III was subjected to PAGE. Measurement of the activity eluted from the gel slices revealed that it was recovered at a position corresponding to the protein (Fig. 3).

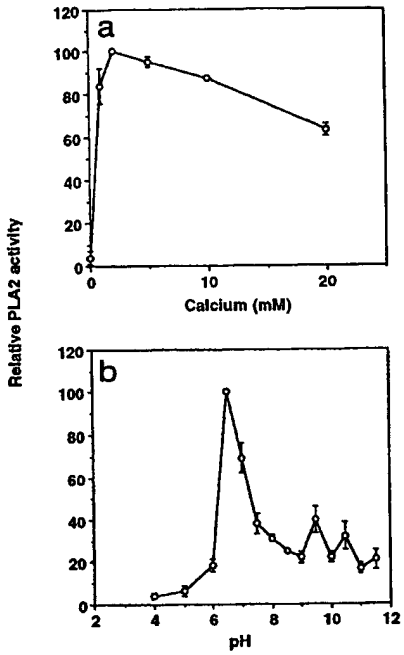

FIG. 4. **Calcium requirement and pH optima.** The dependence of seminal PLA_2 on calcium concentrations (as $CaCl_2$) (*a*) and pH (*b*) in the reaction media was determined as described under "Experimental Procedures." To generate 0 mM Ca^{2+}, 100 μM EGTA was added to calcium-free buffer A. Results are expressed as a percentage of the untreated enzyme and represent the means ± S.E. of three independent experiments.

Calcium Requirement and pH Optimum—In a manner similar to most phospholipases characterized thus far, the enzyme was calcium-dependent and was maximally active at ~2 mM calcium (Fig. 4*a*), while analysis of the pH dependence of the activity revealed a single activity maximum at pH 6.5 (Fig. 4*b*).

Sensitivity of Bovine Seminal PLA_2 to Known PLA_2 Inhibitors—Purified PLA_2 was resistant to pBPB, whereas the two positive controls, porcine pancreatic and *C. atrox* PLA_2, were inhibited (Fig. 5*a*). Seminal PLA_2 was inhibited by gossypol at inhibitor concentrations higher than those required to inhibit crotal PLA_2, but similar to those required to inhibit the porcine pancreatic enzyme (Fig. 5*b*). The porcine enzyme and seminal PLA_2 also shared similar sensitivities to the thiol reagent DTT (Fig. 5*c*); the sensitivity of the crotal enzyme toward DTT was not investigated in this study.

Determination of the Enzyme pI—To determine the pI of PLA_2, isoelectric focusing of a partially purified enzyme was performed (Fig. 6). The gel rod was cut into 24 pieces, which were then left to elute in H_2O. The supernatants were assayed for PLA_2 activity, and their pH was measured. Several (n = 8) such experiments revealed a single activity peak at pH 5.6 ± 0.07 (mean ± S.E.). Typical activity recoveries on the order of 10–20% were obtained. The true recovery is expected to be higher since the Triton X-100 concentration in the supernatants (~0.001% final concentration) inhibited the activity of a partially purified fraction by ~50% (data not shown).

Substrate Specificity of Seminal PLA_2—The substrate specificity was studied in the presence of phospholipid micelles consisting of either PC or PE and deoxycholate or Triton X-100. As summarized in Table II, PLA_2 was 2 orders of magnitude more active toward the deoxycholate-containing substrate than toward the Triton X-100-containing substrate or vesicular substrate (data not shown). In the presence of deoxycholate, the enzyme discriminated between the *sn*-2-fatty acid as it was less active toward PC carrying linoleoyl (1111 ± 98) than arachi-

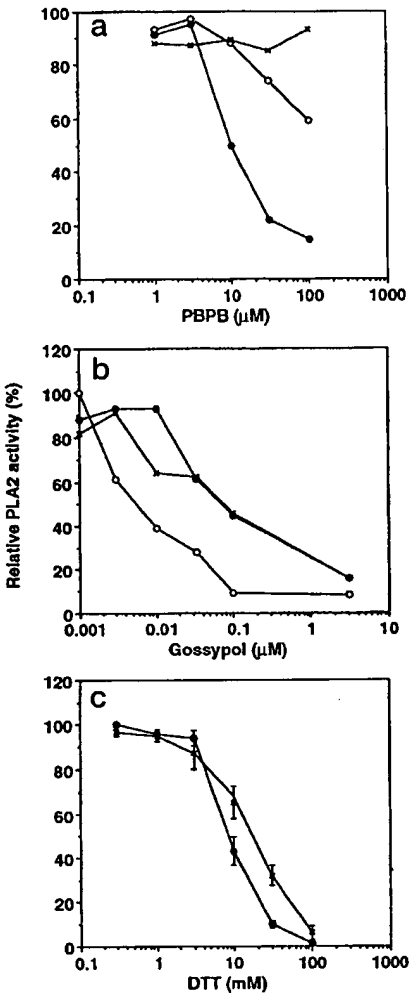

FIG. 5. **Sensitivity of the seminal enzyme to PLA_2 inhibitors.** Seminal (×), crotal (○), and porcine pancreatic (●) PLA_2 were pretreated with the indicated PLA_2 inhibitors as described under "Experimental Procedures." The PLA_2 activity of an aliquot (10-fold diluted) that underwent the appropriate treatment was then assayed over a 30-min period. The data shown here represent the mean of three independent determinations. For clarity, the standard error is shown only in *c*; for *a* and *b*, it was typically below 10% and never above 15% of the corresponding mean.

donyl (1716 ± 73). For a given *sn*-2-side chain, no selectivity was observed between PE/deoxycholate- or PC/deoxycholate-containing micelles as both substrates were hydrolyzed at similar rates, suggesting that the enzyme shows little, if any, head group specificity in this assay system. When micelles comprising Triton X-100 were used, however, head group specificities were observed. The ethanolamine phospholipid was cleaved more efficiently than the corresponding choline phospholipid (56 ± 5.8 *versus* 17 ± 1.5), although the total amount hydrolyzed remained much lower than when deoxycholate was present. Interestingly, the side chain specificity observed with deoxycholate-containing micelles was reversed when Triton X-100 micelles were used, as linoleyl was then preferred over arachidonyl (48 ± 1.7 *versus* 17 ± 1.5).

DISCUSSION

The seminal PLA_2 activity bound specifically to the butyl-Sepharose resin, thus permitting a 130-fold purification in a single step. Choline had to be included throughout this step to prevent the heparin-binding proteins, the main component of bovine seminal plasma (37), from strongly binding to the resin.

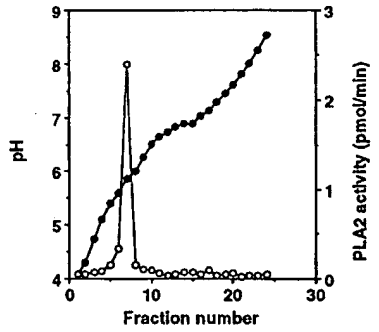

FIG. 6. **Isoelectric focusing of seminal PLA_2.** A partially purified aliquot was adjusted to 5 M urea and 2% ampholytes, applied onto an isoelectric focusing polyacrylamide gel rod, and subjected to isoelectric focusing. The gel rod was then cut into 24 equally sized pieces, which were incubated for 16 h in distilled water. The PLA_2 activity content of the supernatant was then determined. The pH in each fraction was measured using a glass electrode. ●, pH; ○, PLA_2 activity.

TABLE II
Substrate specificity

The partially purified enzyme was incubated with 3-palmitoylphospholipids (0.17 μM) bearing different ^{14}C-labeled *sn*-2-acyl groups and containing either ethanolamine or choline as head group. The substrate was prepared 20 min in advance and was diluted 10 times in the assay tube to yield the indicated detergent concentrations. The results represent the means ± S.E. of three independent experiments.

Phospholipid	Enzymatic activity	
	Deoxycholate (1 mM)	Triton X-100 (0.01%)
Arachidonyl-PE	2,000 ± 32	56 ± 5.8
Arachidonyl-PC	1,700 ± 73	17 ± 1.5
Linoleoyl-PC	1,100 ± 98	48 ± 1.7

Rechromatography of the unadsobed fraction did not permit further binding of the activity, thus suggesting the presence of another form of PLA_2, which was not further investigated in this study. Chromatography on both gel filtration and ion-exchange resins (Fig. 1, *b* and *c*) resulted in activity and protein absorbance patterns that eluted closely together, indicating that the major protein (absorbance at 280 nm) was also responsible for the activity. When analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue, a major 60-kDa band was visible in both chromatographic runs (Fig. 2). Further confirmation that the 60-kDa band was responsible for the activity was obtained by PAGE. Fractions that consisted of eluates of gel slices were assayed for PLA_2 activity, and again, the band intensity and the corresponding enzymatic activity variations matched closely (Fig. 3). Gel filtration revealed that the activity behaved as a 100-kDa protein (Fig. 1*b*), whereas SDS-PAGE analysis showed a 60-kDa band (Fig. 2). This discrepancy might be attributed to dimerization of the 60-kDa enzyme. This dimer appears stable since moderately stringent conditions (0.1% deoxycholate or 5 M urea) failed to shift the elution position of PLA_2 (data not shown). Since the omission of 2-mercaptoethanol did not change its behavior on SDS-PAGE (data not shown), it appears that the interaction is noncovalent. Consistent with the dimer hypothesis, the enzyme behaves on native PAGE as a much larger protein than bovine serum albumin despite a very similar pI (Fig. 3).

Binding to Q-Sepharose at pH 7.4 (Fig. 1*c*) as well as isoelectric focusing (Fig. 6) indicate that the enzyme is acidic. In comparison, most mammalian $sPLA_2$ are neutral to basic proteins, with one notable exception (10). $cPLA_2$, on the other hand, possess pI values similar to those of the seminal enzyme (Fig. 6) (8, 35). Besides this similarity, however, the seminal enzyme shares little in common with $cPLA_2$. Using two different assay systems, the seminal enzyme did not show the char-

acteristic specificity for arachidonylphospholipids found in high molecular mass PLA_2. In the Triton X-100 assay system, the seminal plasma PLA_2 activity toward *sn*-2-arachidonyl was ~3-fold lower than the activity toward linoleyl, whereas $cPLA_2$, in a similar assay system, displayed a 3-fold higher activity (5). Moreover, while $cPLA_2$ is inhibited by deoxycholate micelles relative to sonicated vesicles (4), the reverse is observed for the seminal enzyme (data not shown).

The resistance of the enzyme to pBPB supports the view that this enzyme is novel. pBPB inactivates $sPLA_2$ by alkylating a histidine residue located in the active site of the enzyme (38). It also inactivates $cPLA_2$ (39) by an unknown mechanism, which is likely to be quite different from $sPLA_2$ since $cPLA_2$ does not possess an active-site histidine (3). At the pBPB concentrations used, both enzyme types should be inactivated, and yet, the seminal enzyme is unaffected. As expected, the two PLA_2 controls, the type I porcine pancreatic and the type II *C. atrox* enzymes, were inactivated (Fig. 5*a*). The greater resilience of the crotal enzyme is most likely due to its tendency to shield its active site through dimerization (40, 41). This raises the possibility that seminal PLA_2 possesses a histidine or some other susceptible residue in its active site, which would be completely shielded from the environment in the absence of substrate and/or Ca^{2+}.

Despite this resistance, some common structural features between pancreatic and seminal PLA_2 are suggested by the inhibition patterns of DTT and gossypol. The pancreatic enzyme is inhibited by gossypol at concentrations very close to those required to inhibit the porcine enzyme (Fig. 5*b*). Although the precise structural modifications induced by gossypol are unknown, the similar concentrations required to inhibit pancreatic PLA_2 and the seminal enzyme suggest some common structural elements. This resemblance appears to be quite specific as the inhibition pattern of the crotal enzyme, which shares strong structural homologies with the pancreatic enzyme (1, 2), is completely different. The shared DTT sensitivities (Fig. 5*c*) further support the view that common features between mammalian $sPLA_2$ and seminal PLA_2 exist. Biochemical characterization revealed that seminal PLA_2 shows catalytic properties common to most $sPLA_2$ identified so far: the enzyme is Ca^{2+}-dependent (Fig. 4*a*) and is optimally active in the neutral to alkaline pH range (Fig. 4*b*) (42).

The substrate selectivity profile of purified PLA_2 is also reminiscent of mammalian $sPLA_2$ (43, 44). For instance, these enzymes are activated by the introduction of negative charges (as with deoxycholate *versus* Triton X-100) in the lipid substrate, most likely due to the accumulation of positive charges near the phospholipid-binding site (45). In the absence of deoxycholate, for a given acyl side chain, they are more active toward the anionic phospholipid PE than toward the zwitterionic phospholipid PC (43).

Beside these catalytic similarities, major structural differences appear to exist between these enzymes. For instance, mammalian $sPLA_2$ are low molecular mass (14–20 kDa) and mostly basic proteins, whereas the seminal enzyme possesses a 60-kDa mass and an acidic pI. Pancreatic PLA_2 and the human seminal/synovial enzyme demonstrate affinity for heparin (46–48), while bovine seminal PLA_2 does not (data not shown). Moreover, $sPLA_2$ are resistant to acidic conditions as relatively good recoveries are routinely obtained following chromatography performed under acidic conditions (49–51), whereas the major PLA_2 activity found in seminal plasma is acid-labile (data not shown).

The seminal enzyme displays a specific activity (under suboptimal conditions) of ~0.01 μmol/min/mg, which is rather low compared with that of low molecular mass PLA_2 (for instance, ~40 and 1500 μmol/min/mg for bovine pancreatic and *Naja naja* venom PLA_2, respectively) or with that of $cPLA_2$ (~0.6 μmol/min/mg) (4). The activity range of these well characterized PLA_2 thus covers 5 orders of magnitude. The resistance of seminal PLA_2 to pBPB (Fig. 5*a*) might indicate that it acts via a different, less efficient catalytic mechanism than the established enzymes. The lower catalytic efficiency of bovine seminal PLA_2 could be required for its proper function in seminal plasma. Alternatively, it could possess some yet undetermined advantages over other types of PLA_2 that would render it better suited to the particularity of the bovine reproductive physiology.

These results differ significantly from those reported previously concerning bovine (30) or human (28, 29, 31, 47, 52) seminal plasma PLA_2. The major human seminal plasma PLA_2 has been found to be identical to the synovial enzyme (31, 32). A minor form that was not recognized by the anti-synovial PLA_2 antibody was also reported (31). In the bovine species, the preliminary characterization of the enzyme published previously (30) did not permit any definitive conclusions to be drawn as to the nature of the seminal enzyme. Two different enzymatic activities were partially purified from seminal vesicle secretions. SDS-PAGE of the most purified fraction showed a doublet migrating as 14–16-kDa proteins. This enzyme may represent a minor PLA_2 form. The human prostate enzyme has also been partially purified and characterized (53). Overall, its biochemical properties appear to be quite distinct from those of bovine seminal plasma PLA_2.

The activities found in bovine, ram, and porcine seminal plasma amount to ~1, 10, and 0.03%, respectively, of the human seminal plasma PLA_2 activity (29), suggesting that qualitative differences might exist between the PLA_2 types found in these species. The structural characterization of the enzyme that is currently underway should reveal the reasons behind these differences.

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EXHIBIT C

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Isolation of a cDNA Coding for L-Galactono-γ-Lactone Dehydrogenase, an Enzyme involved in the Biosynthesis of Ascorbic Acid in Plants

PURIFICATION, CHARACTERIZATION, cDNA CLONING, AND EXPRESSION IN YEAST*

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L-Galactono-γ-lactone dehydrogenase (EC 1.3.2.3; GLDase), an enzyme that catalyzes the final step in the biosynthesis of L-ascorbic acid was purified 1693-fold from a mitochondrial extract of cauliflower (*Brassica oleracea*, var. botrytis) to apparent homogeneity with an overall yield of 1.1%. The purification procedure consisted of anion exchange, hydrophobic interaction, gel filtration, and fast protein liquid chromatography. The enzyme had a molecular mass of 56 kDa estimated by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis and showed a pH optimum for activity between pH 8.0 and 8.5, with an apparent K_m of 3.3 mM for L-galactono-γ-lactone. Based on partial peptide sequence information, polymerase chain reaction fragments were isolated and used to screen a cauliflower cDNA library from which a cDNA encoding GLDase was isolated. The deduced mature GLDase contained 509 amino acid residues with a predicted molecular mass of 57,837 Da. Expression of the cDNA in yeast produced a biologically active protein displaying GLDase activity. Furthermore, we identified a substrate for the enzyme in cauliflower extract, which co-eluted with L-galactono-γ-lactone by high-performance liquid chromatography, suggesting that this compound is a naturally occurring precursor of L-ascorbic acid biosynthesis *in vivo*.

Vitamin C or ascorbic acid (L-AA)[1] is an important metabolite for most living organisms present in millimolar concentrations and is well known for its antioxidant properties. Its precise functions in plants is still poorly understood, although it is known to play an important role in the antioxidant system that protects plants from oxidative damage resulting from biotic and abiotic stresses as well as being a cofactor for a number of hydroxylase enzymes.

L-AA is synthesized by all higher plants and by nearly all higher animals except humans, other primates, guinea pigs, bats, and some birds (1–3). L-AA has also been reported to be present in a number of yeasts (4), but several reports suggest that L-AA analogues, rather than L-AA, are present in microorganisms (5–7).

The biosynthesis of L-AA follows different pathways in the animal and the plant kingdom. In animals, D-glucose serves as the first committed precursor in the biosynthesis of L-AA and the last step in the pathway is catalyzed by a microsomal L-gulono-γ-lactone oxidase (EC 1.1.3.8), which oxidizes L-gulono-γ-lactone (L-GuL) to L-AA. This enzyme has been isolated and characterized from rat, goat, and chicken (8, 9).

Despite the importance of L-AA in plants, the biosynthetic pathway has still not been established, although current evidence suggests the existence of two discrete routes. A biosynthetic pathway from D-galactose proceeding via L-galactono-γ-lactone (L-GL) has been proposed as long ago as 1954 by Isherwood *et al.* (10) and Mapson *et al.* (11), based on initial studies of the oxidation of L-GL to L-AA by the enzyme L-galactono-γ-lactone dehydrogenase (GLDase). GLDase activity has been described (11–13) in plants such as pea, cabbage, cauliflower florets, and potato, and recently Ôba *et al.* (14) reported a purification of this enzyme from sweet potato roots. Loewus (15) has proposed an alternative pathway in which L-AA is synthesized from D-glucose via L-sorbosone. The presence of an enzyme able to convert L-sorbosone to L-AA with concomitant reduction of NADP was demonstrated in bean and spinach leaves (16, 17). Conceivably, these distinct routes might be present in different subcellular compartments or in different plant species.

Here, we report the purification and characterization of GLDase from cauliflower florets, followed by isolation and sequencing of the corresponding cDNA. This is the first description of a gene coding for an enzyme involved in the biosynthesis of L-AA in plants. The GLDase cDNA has furthermore been expressed in an active form in yeast, and we have strong indications that the substrate for GLDase, L-GL is naturally present in plant extracts. These findings emphasize for the first time the physiological relevance of the biosynthetic pathway proposed by Isherwood *et al.* and Mapson *et al.* (10, 11).

EXPERIMENTAL PROCEDURES

Materials—Sephacryl SF-200, DEAE-Sepharose, and phenyl-Sepharose CL-4B were obtained from Pharmacia (Uppsala, Sweden). L-Galactono-γ-lactone, D-galactono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z97060.

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[1] The abbreviations used are: L-AA, L-ascorbic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; L-GL, L-galactono-γ-lactone; L-GuL, L-gulono-γ-lactone; GLDase, L-galactono-γ-lactone dehydrogenase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

D-gluconic acid, and *p*-hydroxymercuribenzoic acid were from Sigma. D-Erythronic-γ-lactone, D-xylonic-γ-lactone, and *N*-ethylmaleimide were purchased from Aldrich. Restriction enzymes were from Pharmacia and [α-^{32}P]dCTP was from Amersham (Aylesbury, United Kingdom). Cauliflowers (*Brassica oleracea*, var. botrytis) were obtained from a field nearby Gent and kept at 4 °C until use.

Extraction—Cauliflower florets (7.5 kg) were cut into small pieces and homogenized in a pre-cooled blender in ice-cold buffer A (400 mM sucrose, 100 mM sodium phosphate buffer, pH 7.4) at 1 liter/kg fresh weight. The homogenate was passed through four layers of Miracloth tissue (Calbiochem-Novabiochem, La Jolla, CA), and centrifuged at 13,500 × *g* for 45 min in a GS3 rotor. The pellet containing the mitochondria (approximately 250 g of material) was stored at −70 °C until further use. The crude, frozen mitochondrial pellet was gently thawed in a microwave oven and resuspended in 1/10 volume (750 ml) of buffer A. Cold acetone (−20 °C) was slowly added while stirring (10 × volumes) and the mixture was allowed to stand for 30 min at 4 °C. Precipitated proteins were then collected by filtration through pre-filter paper (A15; Millipore, Bedford, MA) and resuspended in 1/10 volume of buffer B (40 mM Tris-HCl, pH 9.0) followed by 5 h dialysis against 10 volumes of buffer B. The denatured proteins were removed by centrifugation (10,000 × *g* for 15 min). GLDase was then purified from the supernatant and designated as the protein extract, using the protocol described below ("Enzyme Purification"). All manipulations concerning the preparation of extracts and enzyme purification were carried out at 4 °C, unless stated otherwise.

GLDase Assay—GLDase activity was measured spectrophotometrically by following the L-GL-dependent reduction of cytochrome *c* at 550 nm and 22 °C. The reaction mixture (1 ml) consisted of enzyme extract, cytochrome *c* (1.5 mg/ml), and L-GL (4.2 mM) in 0.05 M Tris-HCl buffer (pH 8.4). Under these conditions the reaction rate was linear with respect to time for an initial period of at least 15 min. One unit of enzyme activity was defined as the amount that oxidized 1 μmol of L-AA/min. This corresponds to the reduction of 2 μmol of cytochrome *c* as described by Ôba *et al.* (13). Substrate specificity assays were carried out as described above using 4.2 mM of the different substrates to be tested.

Enzyme Purification—The protein extract (from 250 g of mitochondrial pellet) was loaded onto a DEAE-Sepharose column (5 × 12 cm) equilibrated with buffer B. After washing with 4 column volumes of buffer B at 60 ml/h, elution was carried out with 0.5 M NaCl in the same buffer. Fractions of 8 ml were collected at a flow rate of 60 ml/h, and fractions containing GLDase activity were pooled and ammonium sulfate was added to a concentration of 1 M. The extract was then loaded on a phenyl-Sepharose CL-4B column (2.2 × 15.0 cm) equilibrated in buffer C (1 M ammonium sulfate, 25 mM sodium phosphate, pH 7.0). After washing with 2 column volumes of buffer C, elution was carried out at 30 ml/h by mixing buffer C with a 600-min linear gradient of 80% ethylene glycol in 25 mM sodium phosphate (pH 7.0).

Fractions containing GLDase activity were again pooled, concentrated to 10 ml by ultrafiltration using a PM-10 membrane (Amicon, Beverly, MA), and then applied onto a Sephacryl SF-200 gel filtration column (2.6 × 94 cm) equilibrated in buffer D (20% ethylene glycol, 40 mM NaCl, 80 mM sodium phosphate, pH 7.4). The enzyme was eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 5 ml were collected and fractions with activity pooled. This preparation could be stored at 4 °C for several weeks without any detectable loss of activity.

Two gel filtration preparations were combined and concentrated with buffer exchange to buffer E (20% ethylene glycol, 20 mM Tris-HCl, pH 8.0) by ultrafiltration (PM-10 membrane). The resulting solution was applied to a strong anion exchange column (Resource Q, 6 ml; Pharmacia Biotech Inc.) equilibrated in buffer E and connected to an FPLC system (Pharmacia). The column was eluted at 1 ml/min with a gradient of 0–450 mM NaCl in buffer E as follows: 0–85 mM in 18 min, 85–110 mM in 10 min, 110–130 mM in 14 min, and 130–450 mM in 10 min. Fractions of 1 ml were collected. The activity of the main peak, which eluted at 120 mM NaCl, was collected and adjusted to pH 6.0 with 50 mM sodium phosphate.

The pooled fractions were loaded onto a Poros 20 SP strong cation exchange column (PerSeptive Biosystems, Cambridge, MA) equilibrated in buffer F (20 mM sodium phosphate, pH 6.0, 20% ethylene glycol) and eluted using the FPLC at a flow rate of 1 ml/min. Elution was carried out with a gradient of 0–500 mM NaCl in buffer F as follows: 125–225 mM in 40 min and 225–500 mM in 37 min. Fractions of 2 ml were collected. Two peaks of activity eluted: peak I at 210 mM and peak II at 225 mM NaCl. Peak II was dialyzed against 10 mM sodium phosphate, pH 7.2, containing 1 mM L-AA and the volume was reduced to 200 μl by lyophilization (Heto Lab Equipment, Lyngby, Denmark).

As a final step, the pooled fractions of peak II were separated by HPLC using a Zorbax gel filtration column GF-250 (9.4 × 250 mm) (Rockland Technologies Inc., Newport, DE) equilibrated in 750 mM NaCl, 50 mM sodium phosphate (pH 7.2). Fractions of 1 ml were collected at a flow rate of 1 ml/min.

Protein Determination—The protein concentration of extracts was determined according to Bradford (18) using bovine serum albumin as standard.

Determination of Molecular Mass—The molecular mass of the native GLDase was estimated by gel filtration on a Sephacryl SF-200 column (2.5 × 94 cm) equilibrated in 40 mM NaCl, 80 mM sodium phosphate (pH 7.4). The column was eluted at a flow rate of 20 ml/h and fractions of 4 ml were collected. The molecular mass was estimated by comparing the elution of GLDase with that of the standard proteins: ferritin (450 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.5 kDa).

SDS-PAGE—Analytical SDS-PAGE was performed in slab gels of 10% polyacrylamide as according to Chua (19). Proteins were visualized either by Coomassie Brilliant Blue R-250 staining (19) or silver nitrate staining (20).

Affinity Retardation Chromatography—Cytochrome *c* was covalently bound to thiol-activated Sepharose 4B as described by Azzi *et al.* (21) and packed into a column (1.0 × 20 cm) that eluted at flow rates of 8 ml/h in 10 mM sodium phosphate buffer (pH 7.4). Fractions of 2 ml were collected and tested for activity.

Lycorine Extraction—Lycorine was purified from non-flowering, whole plants of *Crinum jagus* or *Crinum asiaticum* as described by Davey *et al.*[2].

Partial Amino Acid Sequence Determination—Purified GLDase from the Poros 20 SP purification step was applied to SDS-PAGE. The separated polypeptides were blotted onto polyvinylidene difluoride membranes (Millipore) as described by Bauw *et al.* (23). NH_2-terminal and internal amino acid sequence analyses of the polyvinylidene difluoride-bound proteins were performed as described by Bauw *et al.* (24). Trypsin was used for the *in situ* digests and the resulting peptides were separated by reversed-phase HPLC. Amino acid sequencing was performed on a 473 protein sequencer (Applied Biosystems, Foster City, CA).

Isolation of Total RNA and First-strand cDNA Synthesis—Cauliflower floret tissue (300 mg) was ground to a powder in liquid nitrogen with a mortar and pestle and RNA was extracted using a method based on LiCl precipitation as described by Goormachtig *et al.* (25). The RNA isolated from cauliflower florets (4 μg) was used to synthesize first-strand cDNA according to the instruction manual for Superscript™ Preamplification System for first-strand cDNA synthesis (Life Technologies, Inc., Gaithersburg, MD).

Polymerase Chain Reaction—Degenerate oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems) and used as primers in polymerase chain reactions. The peptide sequences used for synthesizing the corresponding coding and complementary oligonucleotides were designed according to the partial amino acid sequence obtained earlier, and designated 3, 6, and 8 (*underlined* in Fig. 5).

First-strand cDNA synthesized from cauliflower florets was used as a template. The amplification mixture consisted of template, polymerase chain reaction buffer, 200–300 ng of each primer, 2.5 mM cNTP, and 1 unit of *Taq* polymerase in a total volume of 50 μl. The amplification program consisted of 32 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 1 min), and primer extension (72 °C, 2 min). Products of the reaction were separated on 1% agarose gels, excised, and then purified according to the QIAEX Handbook (Diagen GmbH, Hilden, Germany). The purified products were cloned into a pGEM-T vector (Promega, Madison, WI).

Screening of cDNA Library—A cauliflower cDNA library constructed in λZAP II (Stratagene, La Jolla, CA) was used. Aliquots of the cDNA library were plated out using *Escherichia coli* XL-1 Blue cells on 23 × 23-cm baking plates (Nunc, Roskilde, Denmark) containing NZY agar. Approximately 600,000 plaques of the library were transferred onto duplicate nylon membranes (Hybond N^+; Amersham). The membranes were treated in accordance with the manufacturer's instructions for plaque blotting. DNA was fixed to membranes by irradiation with ultraviolet light (UV Stratalinker; Stratagene). A 250-bp polymerase chain reaction-amplified fragment was labeled with [α-^{32}P]dCTP using a random primed DNA labeling kit (Boehringer, Mannheim, Germany)

[2] M. W. Davey, G. Persiau, A. De Bruyn, J. Van Damme, G. Bauw, and M. Van Montagu, submitted for publication.

TABLE I
Purification scheme for GLDase
Mitochondrial extract from 15 kg of cauliflower florets were used for the preparation

Step	Volume	Protein	Activity		Fold	Recovery
			Total	Specific		
	ml	*mg*	*units*	*units/mg*		%
Acetone precipitation	2,500	1,510	44,900	30	1	100
DEAE ion exchange	83	55	46,500	845	28	104
Phenyl-Sepharose	38	21	30,800	1,467	49	69
Gel filtration	54	11	20,900	1,900	63	47
FPLC Resource Q	32	0.3	8,100	27,000	900	18
FPLC Poros 20 SP	4	0.01	508	50,800	1,693	1.1

and subsequently used as probe for screening the cDNA library. The membranes were washed for 4 h at 65 °C in hybridization buffer (1% (w/v) bovine serum albumin, 7% (w/v) SDS, 1 mM EDTA, and 0.25 M sodium phosphate, pH 7.2), before 20 h incubation with the ^{32}P-labeled probe in hybridization buffer at 65 °C. The membranes were then rinsed twice for 15 min with 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0) and 1% SDS at room temperature and exposed to X-Omat AR film (Kodak, Rochester, NY) with an enhancer screen for autoradiography. Plaque-purified phage clones were converted into phagemids (Bluescript SK-/+; Stratagene) by *in vivo* excision using the ExAssist™ System.

DNA Sequence Determinations—DNA sequence determinations were carried out in accordance with protocols obtained from Applied Biosystems. Initial sequences were obtained by use of T7 and T3 vector primers. To complete the sequences on both strands, cDNA-specific primers were used. The sequence analyses were carried out using software of the Genetics Computer Group (Madison, WI).

Expression in Yeast—To express the GLDase cDNA in yeast (*Saccharomyces cerevisiae*), the Bluescript vector containing the full-length cDNA was digested with *ApaI* and *KpnI* and a 27-bp adapter containing a *NotI* restriction site subsequently ligated into the *ApaI-KpnI*-linearized vector. The resulting construct containing two *NotI* restriction sites was cloned into the *NotI* restriction sites of the pFL61 vector (26). Yeast cells of the strain W303B (*Mata, ade2, ura3, his3, trp1, leu2, can*1-100) (27) were transformed by the method of Dohmen *et al.* (28) and plated on selective 1.5% agar plates (lacking uracil) containing minimal SD medium (0.2% yeast nitrogen base (Difco, Detroit, MI), 0.7% ammonium sulfate, 2.7% glucose) supplemented with adenine, tryptophan, leucine at 20 μg/ml, and histidine at 10 μg/ml (as above minus agar). Transformed cells were transferred to liquid SD medium and grown for 3 days at 30 °C. The cells were collected by centrifugation (8,000 × *g*, 15 min), washed, and resuspended in 50 mM Tris-HCl (pH 8.0). For GLDase activity tests and protein determinations the cells were disrupted by two passages through a French Press after a cycle of freezing (−70 °C) and thawing.

Extraction and HPLC Analysis of L-GL—Up to 1 g of plant tissue was first thoroughly homogenized using a pestle and mortar in liquid nitrogen, and extracted using 10% trichloroacetic acid to precipitate proteins and inhibit degradative enzymes. After filtration and partitioning against water-saturated diethyl ether to remove trichloroacetic acid, samples were concentrated and injected onto the C18 HPLC column, eluted with 0.1% trifluoroacetic acid. Peaks eluting in the region of L-GL elution were collected and tested for their ability to serve as a substrate for GLDase. Analogous peaks from up to 10 runs were combined, dried under vacuum, and re-injected onto the aminopropyl HPLC column for weak anion exchange. Once again peaks eluting in the region of the L-GL standard were collected and tested for their ability to serve as a substrate for GLDase. Positive peaks from several runs were pooled, concentrated, and finally reinjected on a C18 reversed-phase HPLC column eluted with phosphoric acid (pH 2.5). HPLC was carried out using a 600E pump (Waters, Milford, MA) and a Waters 996 diode-array detector. Injections (20–40 μl) were made using a WISP 412 (Waters) autosampler onto a C18, 3-μM spherical particle size, 250 × 4.6 mm inner diameter, reversed-phase HPLC column (Bio-Rad), fitted with a 10-mm guard column. Separations were carried out isocratically at 800 μl/min with phosphoric acid (pH 2.5), or 0.1% trifluoroacetic acid as mobile phase. Data were collected and analyzed, and the entire system was controlled using the Millenium 2010 (v1.15) chromatography management system (Waters). Weak anion exchange separations were carried on a 250 × 3.6-mm aminopropyl column (Phenomenex Inc., Torrance, CA), eluted isocratically with 15% (v/v) 20 mM KH_2PO_4 (pH 6.0), in acetonitrile. The column was regenerated after each analysis with a 10-min linear gradient of 15–50% acetonitrile in 20 mM KH_2PO_4 (pH 6.0) at 1 ml/min. Strong anion exchange HPLC with pulsed amperometric electrochemical detection was carried out on the same system fitted with an HP 1049A electrochemical detector containing a gold amalgam-working electrode at an operating potential of +100 mV. Separations were performed on a 300 × 4.6-mm, Dionex PA-100 strong anion exchange column (Dionex Corp., Sunnyvale, CA) eluted with a 20-min linear gradient of 0 to 200 mM sodium acetate in 3 ml/liter NaOH.

RESULTS

Enzyme Purification

A summary of the purification of GLDase from cauliflower florets is presented in Table I. As the enzymatic activity was found to be most stable in 20% ethylene glycol this reagent was included in all buffers except for buffers A and B used in the first two purification steps. Interestingly, after the DEAE-Sepharose step the total GLDase activity increased slightly, probably due to removal of inhibitory compounds present in the crude extract. The first three purification steps had relatively little influence on the purity of GLDase, but the FPLC Resource step (strong anion exchange) resulted in an increase in the purification factor from 63 to 900, although there was a corresponding decrease in recoveries to only 47% compared with the activity present in the gel filtration pool. After passage through the strong cation exchange column (Poros 20 SP), GLDase activity was resolved into two peaks designated I and II (Fig. 1). The activity forming the latter peak was used for further analysis. At this stage GLDase was purified 1693-fold from the initial mitochondrial fraction with a recovery of 1.1% (Table I). The purity of the final enzyme preparation was confirmed by SDS-PAGE, where we consistently obtained three polypeptide bands corresponding to approximately 56, 30, and 26 kDa (Fig. 2). Further purification of the enzyme by a high resolution gel filtration on a Zorbax GF 250 column did not result in elimination of the 30- and 26-kDa polypeptide bands; and subsequent amino acid sequence analyses revealed them to be breakdown products of the 56-kDa band. The native molecular mass of the enzyme was estimated to be approximately 56 kDa by Sephacryl SF-200 (Fig. 3) and Zorbax GF 250 high resolution gel filtration.

Partial Amino Acid Sequence Determination of Purified GLDase Polypeptides

NH_2-terminal sequence analysis of the complete 56- and 30-kDa polypeptide bands were found to be identical, and the partially determined sequence of the 26-kDa band was located within the deduced amino acid sequence of the GLDase cDNA (Asp-273 to Leu-289). Trypsin digestions of the 56-kDa protein yielded a series of peptides which were separated by reversed-phase HPLC. A number of the peptides were subjected to partial sequence analysis and could again be located in the GLDase cDNA, as indicated in Fig. 5.

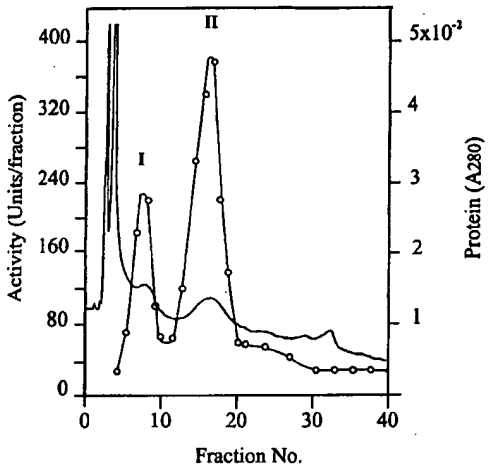

FIG. 1. **Separation and purification of GLDase activity (○) peak I and peak II by a Poros SP cation exchange column.** Protein (A_{280}) (—).

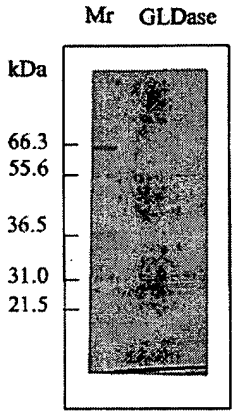

FIG. 2. **SDS-PAGE.** *Lane A*, molecular mass standards; *lane B*, GLDase peak II from the Poros SP (strong anion exchange) purification step, analyzed by SDS-PAGE after an additional high-resolution HPLC gel filtration step. A polypeptide band corresponding to approximately 56 kDa (GLDase), and two degradation products of 30 and 26 kDa (confirmed by amino acid sequence analyses) were visualized by silver nitrate staining.

Characterization

Substrate Specificity and pH Dependence—Various isomeric compounds were tested as possible substrates for the purified GLDase using cytochrome *c* as electron acceptor. These were L-GL, D-galactono-γ-lactone, D-gulono-γ-lactone, L-GuL, D-erythronic-γ-lactone, D-xylonic-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. Apart from L-GL, none of the compounds tested could serve as a substrate for GLDase because no reduction of cytochrome *c* was observed.

GLDase obeyed Michaelis-Menten-type kinetics using L-GL as substrate. With the method of Lineweaver and Burk (Fig. 4), the K_m value was determined to be 3.3 mM with a V_{max} of 7.1 units/min. Concentrations of L-GL used were from 1.0 to 32.6 mM. Substrate inhibition was observed at 32.6 mM.

The pH dependence of the enzyme activity was examined using 50 mM sodium phosphate buffer in the pH range from 6.0 to 7.6 and 50 and 100 mM Tris-HCl in the range between 7.4 and 8.8 at 22 °C with 4.2 mM L-GL. A broad maximum of activity between pH 8.0 and 8.5 was observed (results not shown).

Electron Acceptors—The enzyme assay is based on the reduction of cytochrome *c* by GLDase, in which for each micromole of oxidized L-GL, 2 μmol of cytochrome *c* are reduced, because the L-AA formed is spontaneously oxidized by cytochrome *c* to dehydroascorbic acid. The purified GLDase showed strict specificity for cytochrome *c*, and neither FAD, NAD, NADP, nor molecular oxygen were able to serve as electron acceptors for the enzyme.

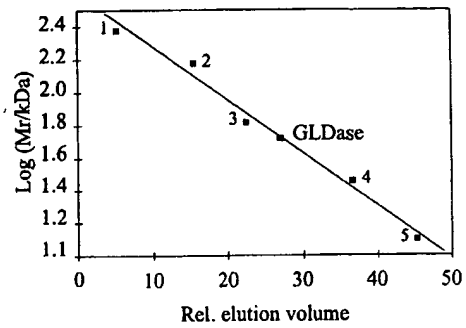

FIG. 3. **Estimation of molecular mass of GLDase.** The native molecular mass was estimated by gel filtration chromatography on Sephacryl SF-200. The *arrow* indicates GLDase activity. Molecular mass standards used were: *1*) ferritin (450 kDa); *2*) alcohol dehydrogenase (150 kDa); *3*) bovine serum albumin (66 kDa); *4*) carbonic anhydrase (29 kDa); and *5*) cytochrome *c* (12.5 kDa).

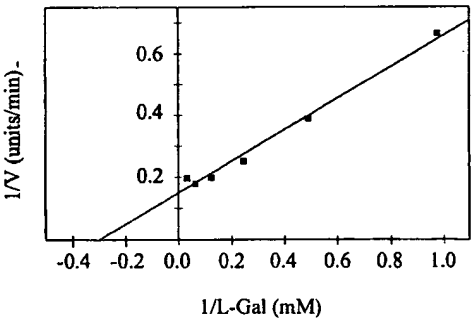

FIG. 4. **Lineweaver-Burk plot of GLDase activity as a function of L-galactono-γ-lactone concentration.**

Inhibitors/Stimulators—The effect of various substrate analogues, organic inhibitors, and some divalent metal ions were examined for their influence on the enzyme activity. The oxidation of L-GL by GLDase was tested in the presence of equimolar concentrations of each of the following compounds: D-galactono-γ-lactone, D-gulono-γ-lactone, L-gulono-γ-lactone, D-erythronic-γ-lactone, D-xylonic-γ-lactone, L-mannono-γ-lactone, D-galactonic acid, D-glucuronic acid, and D-gluconic acid. None of these had any influence on the reaction rate.

Of the divalent metal salts we tested, $MgCl_2$, $CaCl_2$, and $SrCl_2$ had no effect on the GLDase activity at concentrations up to 15 mM. The chelating agent EDTA had no significant effect on the enzyme activity supporting the conclusion that there was no metal requirement for the enzymatic activity.

Sulfhydryl-modifying agents, however, were able to partially inhibit GLDase: *N*-ethylmaleimide, monoiodoacetic acid, and *p*-hydroxymercuribenzoic acid inhibited the enzyme activity by 18% at 12.5 mM, 42% at 26.9 mM, and 81% at 0.4 mM, respectively. These observations indicate that cysteine residues play an important role in the enzyme catalysis. We did not observe any inhibition of the GLDase-dependent reduction of cytochrome *c* in the presence of 5.2 mM riboflavin, a well known flavoprotein inhibitor (29–31).

Lycorine, an alkaloid isolated from members of the *Amaryllidaceae* has been reported to be a specific inhibitor of ascorbic acid biosynthesis in plants and animals at concentrations as

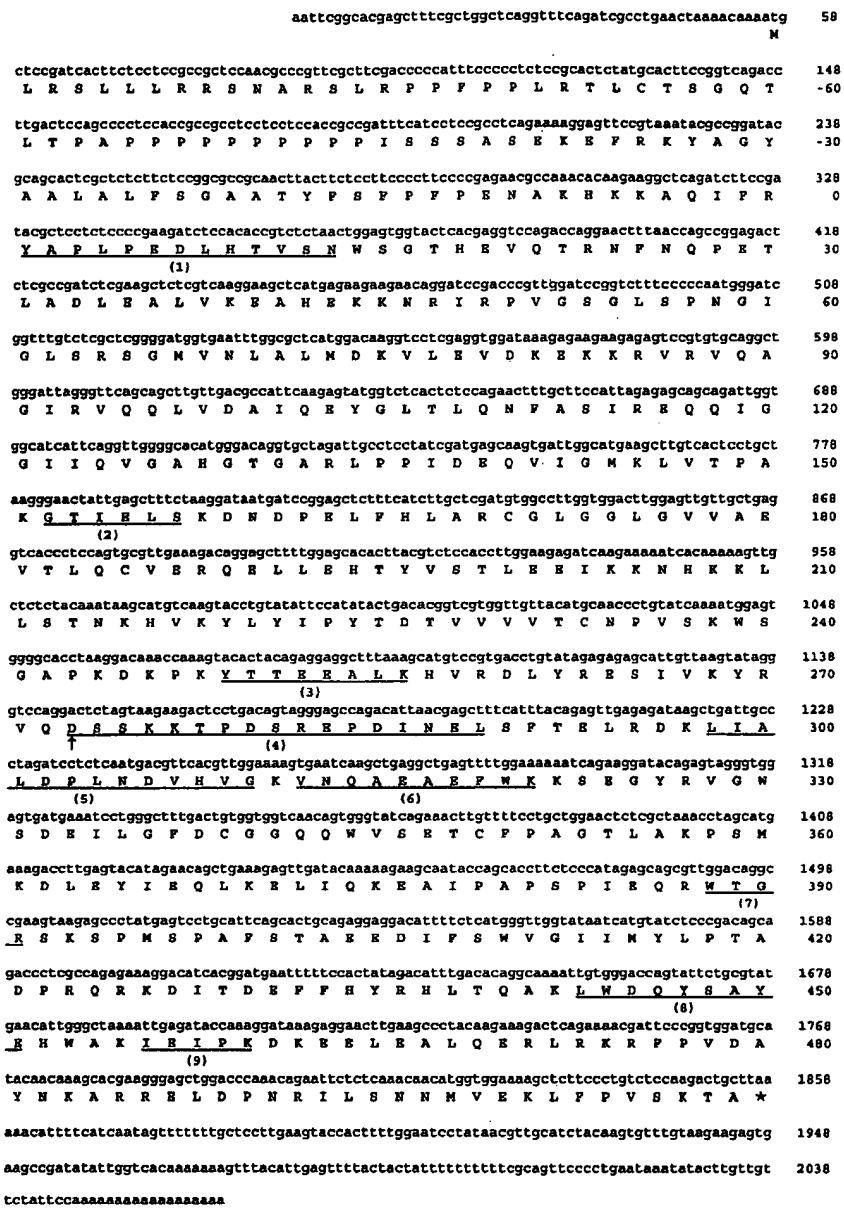

FIG. 5. **Nucleotide sequence and predicted amino acid sequence of GLDase.** Nucleotides are numbered from the first base of the cDNA insert. The deduced amino acid sequence is indicated below the nucleotide sequence in single-letter code. The first methionine of the open reading frame is designated as the first amino acid of the putative polypeptide. The termination codon is indicated by an *asterisk*. Amino acid sequences determined from GLDase polypeptides are *underlined* and *numbered 1–9*. Degenerate oligonucleotides were designed based on peptides 3, 6, and 8. "↕" indicates the point of breakage forming the 26- and 30-kDa degradation products separated by SDS-PAGE (Fig. 2).

low as 1 μM (32–34); once again, however, no influence of lycorine on GLDase activity could be found at concentrations of up to 100 μM.

Cytochrome c Affinity Chromatography—Partly purified enzyme extract was observed to be slightly retarded compared with other proteins (measured as the absorption at 280 nm) when eluted from a cytochrome *c* affinity column. This indicated interaction between GLDase and cytochrome *c*.

Isolation and Sequencing of GLDase cDNA Clone

DNA fragments were obtained by polymerase chain reaction amplification of oligo(dT)-primed cDNA using degenerate oligonucleotides (based on the peptide sequences) as primers. These DNA fragments were subcloned into a pGEM-T vector and sequenced. One 400-bp fragment contained a nucleotide sequence which corresponded to the amino acid sequence of one of the sequenced internal peptides in addition to the sequences corresponding to the primers. Therefore, this fragment was radiolabeled and used as a probe to screen a cDNA library from cauliflower. We screened 2×10^6 plaques resulting in isolation of several positive clones. After *in vivo* excision of the Bluescript plasmid followed by digestion with *Eco*I and *Kpn*I, the two longest cDNA inserts were found to be approximately 2,000 bp. Subsequent subcloning and sequencing revealed an uninterrupted open reading frame of 1803 nucleotides, containing all of the partially sequenced tryptic peptides, the NH_2-terminal amino acid sequence, the first ATG codon (position 56) representing the consensus sequence of an initiator codon (35), and a TAA terminator codon. The presence of these elements showed that the full-length cDNA corresponding to the purified protein had been isolated. Fig. 5 shows the deduced amino acid sequences of the 1803-bp open reading frame coding for 600 amino acids, a 55-bp putative 5′-noncoding region, and a 206-bp 3′-noncoding region including a poly(A) tail. A hexanucleotide AATAAA consensus signal for polyadenylation is found 20 nucleotides before the poly(A)$^+$ tract. Interestingly, nucleotides coding for the determined NH_2-terminal amino acid sequence were found 270 bp downstream from the initiator codon, indicating that the protein is synthesized as a preprotein (600 amino acids with a predicted molecular mass of

67,829 Da). The resulting mature protein of 509 amino acids has a calculated molecular mass of 57,837 Da and a theoretical pI value of 6.85. A putative mitochondrial signal is also present (36).

Expression in Yeast

The GLDase cDNA was cloned into a pFL61 yeast vector (26) in both the sense and antisense orientations relative to the phosphoglycerate kinase promoter and terminator. Untransformed and transformed yeasts were grown and extracts were prepared and tested for GLDase activity. Extracts from yeast transformed with a sense-oriented GLDase cDNA showed a specific GLDase activity of 3.0 units/min/mg protein compared with those made from extracts from untransformed yeast and yeast transformed with antisense orientated GLDase cDNA in which no GLDase activity could be measured with L-GL as substrate (Fig. 6).

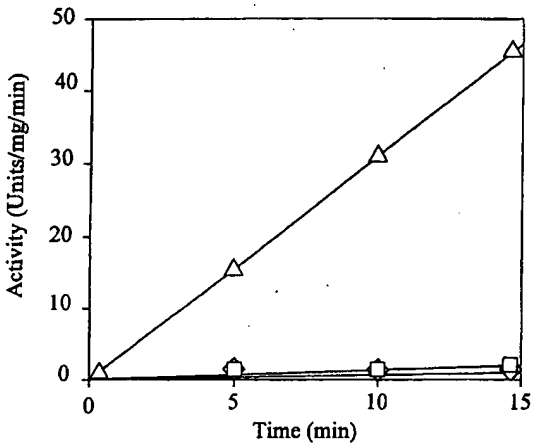

FIG. 6. **Expression in yeast.** GLDase activity in nontransformed yeast (□), and in yeast transformed with the GLDase in sense orientation (△), and in antisense orientation (◇).

HPLC Analysis of L-GL

We used several different systems for the analysis of L-GL by HPLC. These included ion suppression reversed-phase HPLC, weak anion exchange HPLC, and strong anion exchange HPLC. In no case was it possible to obtain unequivocal resolution of L-GL from all other sugar-lactone analogues, but semi-preparative separations using weak anion exchange and reversed-phase HPLC in combination with spectrophotometric assays for GLDase activity, allowed us to consistently identify a fraction that co-migrated with L-GL standard and which served as a substrate for the GLDase-based reduction of cytochrome *c*. Peaks co-eluting in all three systems with L-GL standard were found to be able to serve as a substrate for GLDase (results not shown). This indirect evidence strongly suggests the presence of a natural substrate for GLDase in plant tissue extracts. In addition to this observation, acid extracts of plant tissues were resolved using pulsed amperometric detection and strong anion exchange on a Dionex PA-100 column. Under conditions of high pH (pH 11–12), it is possible to ionize neutral carbohydrates at the C-2 OH position, allowing the separation on appropriate ion exchange columns. Analysis of acid extracts from cauliflower and parsley by strong anion exchange HPLC with pulsed-amperometric detection at a gold electrode showed the presence of small amounts of a peak that co-migrated with L-GL (data not shown). However, in this system, L-GL also co-migrates with DL-GuL and with D-GL, so that it is not possible to unequivocally demonstrate the presence of this compound as a natural substrate.

DISCUSSION

GLDase was purified 1693-fold from cauliflower florets by a 5-step method with 1.1% recovery. The loss in recovery was approximately 20% in each purification step. This compares favorably with the results of Ôba *et al.* (14) who recently published a 5-step purification of GLDase from sweet potato roots in which the enzyme was purified 294-fold with a recovery of 0.9% with a specific activity of 37,000 units/mg. By comparison, after our purification method we obtained a specific activity of 50,800 units/mg.

From the Poros SP column (strong anion exchange) GLDase activity was separated into two peaks of activity (I and II), suggesting the existence of at least two isoforms of GLDase. The most pure and abundant peak, peak II, was subjected to high-resolution gel filtration by HPLC and analyzed by SDS-PAGE (Fig. 2). A polypeptide band corresponding to approximately 56 kDa and two degradation products (confirmed by amino acid sequence analysis) of 30 and 26 kDa separated on the gel.

In most respects, the physical characteristics of GLDase from cauliflower are similar to those of the enzyme purified from sweet potato roots. With regard to the substrate specificity we found like Mapson and Breslow (12) that GLDase was absolutely specific for L-GL; Ôba *et al.* (14), however, observed a 1% oxidation of L-GuL relative to L-GL by the sweet potato enzyme. We measured the K_m value of GLDase for L-GL as substrate to be 3.3 mM which is again in the same range as the value obtained by Mapson and Breslow (12), but considerably higher than the value of 0.12 mM obtained by Ôba *et al.* (14).

However, the native molecular mass of 56 kDa determined by gel filtration is identical to the value obtained by Ôba *et al.* (14). According to our experiments, GLDase has a pH optimum between 8.0 and 8.5, which again corresponds well with the results obtained by Mapson and Breslow (12) and Ôba *et al.* (14).

The GLDase enzyme from cauliflower seems to require sulfhydryl groups for its activity, as reduced activity was observed in the presence of reagents which inactivate these groups. Strongest inhibition was observed with 0.4 mM *p*-hydroxymercuribenzoic acid which caused 81% inhibition. These observations are in accordance with results obtained by Mapson and Breslow (12), who obtained 50% or more inhibition with all sulfhydryl group-modifying agents tested.

Arrigoni *et al.* (37) recently published results from which they concluded that the alkaloid lycorine acts by inhibiting the conversion of L-GL to L-AA. Consequently, the enzyme we have purified from cauliflower is different to the homologous enzyme which Arrigoni *et al.* (37) used for their measurements. These authors also isolated GLDase activity from cauliflower using a different protocol including detergent. We were unable to detect any influence of lycorine on the activity of GLDase from cauliflower at concentrations of up to 100 μM lycorine.

Based on the partial amino acid sequences of tryptic peptides, the cDNA for GLDase was cloned and characterized. The complete amino acid sequence deduced from the cDNA and the localization of the NH_2-terminal amino acid sequence suggest that the mature GLDase protein is preceded by a 91-amino acid pre-peptide. We consider GLDase from cauliflower to be a mitochondrial enzyme as it was purified from a mitochondrially enriched extract from cauliflower florets. This corresponds well with the fact that the deduced pre-protein contains a relatively high number of Ala, Leu, Arg, and Ser residues (11, 10, 8, and 10, respectively); and relatively few Asp, Glu, Ile, and Val residues (0, 3, 2, and 0, respectively), which is characteristic for polypeptides targeted to the mitochondria (38, 39). In addition, the GLDase pre-protein cleavage site FR↓YA resembles a

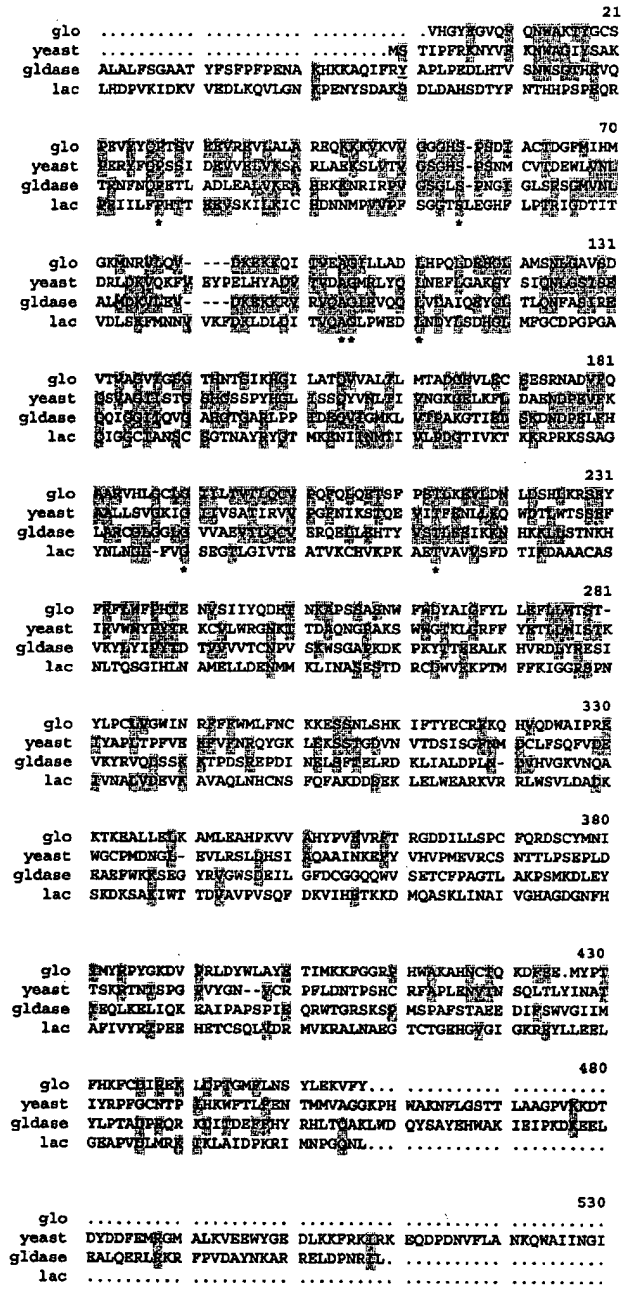

FIG. 7. **Sequence comparison.** Alignment of the amino acid sequence (residue −29 to 509) of GLDase (*gldase*) with the amino acid sequence of L-gulono-γ-lactone oxidase from rat (*glo*) (48), a sequence from the *S. cerevisiae* sequencing project (yeast),[3] and D-lactate ferricytochrome *c* oxidoreductase from *S. cerevisiae* (*lac*) (22). *Dashes* represent regions where the sequences have been extended to allow optimal sequence alignment. Identical residues are indicated by *shaded letters*. *Asterisks* indicate identical residues present in all four sequences. Indicated *numbers* on the *right* refer to the amino acid positions used for GLDase in Fig. 5.

cleavage site motif (RXY ↓ (S/A) which is relatively common in a number of higher and lower eukaryotes (36). These data are in accordance with results obtained by Ôba *et al.* (13) who by sucrose density gradient cell fractionation of extract from potato tuber tissue detected GLDase activity in the same fractions as fumarase, a mitochondrial marker enzyme. By the same technique, Mutsuda *et al.* (40) judged the enzyme to be located in mitochondrial membranes of spinach leaves. The relative molecular mass of GLDase was predicted to be 57,837 Da from the cDNA, which corresponds well with the molecular mass of 56 kDa, estimated by SDS-PAGE and gel filtration. We found one potential *N*-glycosylation site Asn-*X*-Ser/Thr at position 13 in the mature protein, but the enzyme was unretarded on a lectin concanavalin A column, which binds molecules containing an α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related sugars (41).

It has been suggested that GLDase contains a covalently bound flavin moiety as prosthetic group, because inhibition of GLDase activity by flavoprotein inhibitors was reported by Mapson and Breslow (12) and Ôba *et al.* (14). However, we could not observe any inhibition of GLDase from cauliflower by riboflavin or obtain the typical flavin protein absorption spectrum from the purified GLDase. In addition, there was no significant homology between the deduced amino acid sequence of cauliflower GLDase and the sequences of flavin-binding regions of the following flavoenzymes: rat liver dimethylglycine dehydrogenase (42), beef heart succinate dehydrogenase (43), *E. coli* succinate dehydrogenase (44), *E. coli* fumarate reductase (45), *Arthrobacter oxidans* 6-hydroxy-D-nicotine oxidase (46), and rat liver NADPH-cytochrome P-450 oxidoreductase (47).

Interestingly, an homology search with cauliflower GLDase in protein data bases revealed that the first 230 NH_2-terminal amino acid of GLDase had 28% identity with an NH_2-terminal stretch of L-gulono-γ-lactone oxidase (EC 1.1.3.8) from rat (48), 26% with a yeast clone,[3] and 16% with an NH_2-terminal stretch of D-lactate ferricytochrome *c* oxidoreductase from yeast (22) (Fig. 7). This conservation between the NH_2-terminal parts of L-gulono-γ-lactone oxidase and GLDase could indicate that this domain is involved in similar functional roles.

Here, we have clearly shown that the isolated GLDase is 100% specific for L-GL and that a peak that co-elutes with L-GL in four different HPLC separation systems, and can serve as a substrate for GLDase, is naturally present in plant extracts. Although we cannot unequivocally rule out the presence of some other compound, the high substrate specificity of GLDase strongly suggests that L-GL is a natural constituent of plant tissues and that this pathway of L-AA biosynthesis is physiologically relevant.

The results presented here are the first important step in the characterization of the biosynthetic pathway of L-AA in plants. In our laboratory we are progressing toward making plants transformed with the GLDase cDNA, which will allow us to down-regulate the expression of GLDase by antisense technology and study the role of L-AA in plants. Furthermore, in the near future the isolation of the GLDase cDNA may give us the possibility to engineer crops containing stably increased levels of vitamin C.

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